

## For Reference

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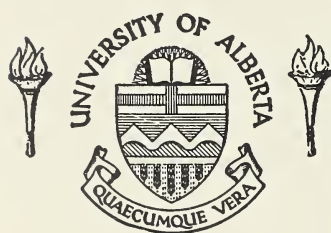
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THE SEROLOGICAL INVESTIGATION  
OF  
SYPHILIS AND TUBERCULOSIS

by

Marguerite Epp, B.Sc.

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PLATE I



CENTRIFUGAL FREEZE DRYER

THE UNIVERSITY OF ALBERTA

THE SEROLOGICAL INVESTIGATION  
OF  
SYPHILIS AND TUBERCULOSIS

A DISSERTATION  
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF MASTER OF SCIENCE

FACULTY OF MEDICINE  
DEPARTMENT OF BACTERIOLOGY

by

Marguerite Epp

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## ABSTRACT

In Part I this work is concerned with certain aspects of the sero-diagnosis of Syphilis. The influence on the reacting capacity of normal and syphilitic sera of certain commonly variable factors is studied. The factors investigated are those of transport and storage, such as time and temperature, as well as time and bacterial contamination. Effects of storage at different temperatures on the stability of lyophilized syphilitic sera are also considered. Age and temperature within a normal range were found to cause no alteration in the serological results of most normal sera, whereas syphilitic sera were altered drastically at high temperature (37°C.), many becoming anticomplementary. Even at 2-4°C. such sera seemed to lose their reagin content in a number of days. The presence of bacteria cannot be considered as the usual cause of naturally occurring anticomplementary sera, although artificial contamination of normal sera with S. aureus and B. subtilis and ageing at 37°C. developed the anticomplementary property in a high percentage of cases. A small number of artificially contaminated sera changed serologically from negative to positive. This change cannot be wholly ascribed to the influence of bacteria since the phenomenon was also observed in a few of the uninoculated serum controls. Lyophilization of syphilitic sera seemed to preserve the reagin content in most instances though only tentative conclusions are possible due to the small number available for test. It is suggested that the retention of reactivity is likely to be best at a low storage temperature.

Part II deals with the sero-diagnosis of Tuberculosis. The recently described haemagglutination technique of Boyden was studied. Before this test could be used in a clinical investigation certain technical difficulties had to be overcome.



Factors affecting the technical aspects of the test were studied and are described. Certain modifications of the original method were finally adopted, these consisted in the use of 1:80,000 tannic acid instead of 1:20,000; 0.15 ml. Old Tuberculin (Lederle) (1:100) per millilitre of cell suspension instead of 0.15 mg. P.P.D.; and the substitution of bovine albumin for rabbit serum as a diluent. This modified test was finally applied to the study of human sera in conjunction with the Middlebrook-Dubos haemagglutination method.

Distinctly higher titres were observed with the modified Boyden technique in the tuberculous individuals, particularly in the far advanced and moderately advanced cases. The Middlebrook-Dubos method, however, was positive in a greater number of cases. Both methods gave reactions with certain sera from non-tuberculous individuals, but these reactions were more restricted with the Boyden technique being confined almost entirely to the syphilitic group. Since the tuberculin reaction of these individuals was unknown the specificity of the modified Boyden test could not be assessed. The study of sera from subjects with known exposure to tubercle antigens but no disease did not permit any final conclusions, although the modified tannic acid technique could tentatively be interpreted as somewhat more specific.

The complete lack of correlation between the serum titres determined by the two haemagglutination techniques suggests that different antibodies are measured, or at least, that various possible antibodies are bound to different degrees by the individual antigens.



### ACKNOWLEDGEMENTS

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## CHAPTER I

The first part of the book is devoted to a general survey of the subject. It begins with a definition of the term "philosophy" and a discussion of its history. The author then proceeds to a consideration of the various branches of philosophy, including metaphysics, epistemology, ethics, and political philosophy. He also discusses the relationship between philosophy and other sciences, such as psychology and sociology. The second part of the book is devoted to a more detailed examination of the various branches of philosophy. It begins with a discussion of metaphysics, which is the study of the nature of reality. The author then discusses epistemology, which is the study of knowledge. He then discusses ethics, which is the study of morality. Finally, he discusses political philosophy, which is the study of the nature of government and society. The book concludes with a summary of the main points discussed in the previous chapters.

## TABLE OF CONTENTS



## TABLE OF CONTENTS

|                                                                                                                                                 | Page |
|-------------------------------------------------------------------------------------------------------------------------------------------------|------|
| INTRODUCTION -----                                                                                                                              | 1    |
| <br><u>PART I:</u>                                                                                                                              |      |
| A CRITICAL REVIEW OF THE SERO-DIAGNOSIS OF SYPHILIS TOGETHER WITH<br>SOME NEW STUDIES CONCERNING THE STABILITY OF THE SYPHILITIC ANTI-<br>BODY. |      |
| <br><u>Sero-diagnosis of Syphilis</u>                                                                                                           |      |
| I. <u>Historical Review</u> -----                                                                                                               | 5    |
| II. <u>Evaluation and Standardization of Serological</u><br><u>Tests</u> -----                                                                  | 7    |
| III. <u>Limitations of Evaluation Studies</u> -----                                                                                             | 8    |
| IV. <u>Experimental</u> -----                                                                                                                   | 9    |
| The Influence of Some Physical Factors on the Stability<br>of Normal and Syphilitic Sera.                                                       |      |
| A. <u>Influence of Age and Temperature on Serum</u>                                                                                             |      |
| Introduction -----                                                                                                                              | 10   |
| Materials and Methods -----                                                                                                                     | 14   |
| Results -----                                                                                                                                   | 14   |
| Conclusion -----                                                                                                                                | 18   |
| B. <u>Influence of Bacteria on the Anticomplementary</u><br><u>Activity of Serum</u>                                                            |      |
| Introduction -----                                                                                                                              | 18   |
| Materials and Methods -----                                                                                                                     | 18   |
| Results -----                                                                                                                                   | 19   |
| Conclusion -----                                                                                                                                | 26   |



|                                                                        |    |
|------------------------------------------------------------------------|----|
| C. <u>Stability of Lyophilized Syphilitic Sera of Known Reactivity</u> |    |
| Introduction -----                                                     | 27 |
| Materials and Methods -----                                            | 27 |
| Results -----                                                          | 29 |
| Conclusion -----                                                       | 32 |
| V. <u>Summary of Part I - Sections A, B and C</u> ----                 | 33 |

PART II:

## STUDIES OF THE HAEMAGGLUTINATION REACTION IN TUBERCULOSIS.

|                                                          |    |
|----------------------------------------------------------|----|
| <u>Sero-diagnosis of Tuberculosis</u>                    |    |
| I. <u>Historical Review</u> -----                        | 35 |
| II. <u>Introduction</u> -----                            | 38 |
| III. <u>Investigation of the Haemagglutination Test,</u> |    |
| <u>Boyden's Modification</u> -----                       | 39 |
| 1. Materials and Methods -----                           | 39 |
| 2. Initial Observations -----                            | 40 |
| Time of Centrifugation                                   |    |
| Agglutination Pattern                                    |    |
| 3. Investigation of Individual Factors ----              | 46 |
| (a) Influence of Diluent Serum from                      |    |
| Different Rabbits -----                                  | 46 |
| (b) Influence of Electrolyte Concentra-                  |    |
| tion on the Stability of Tannic acid-                    |    |
| Old Tuberculin-treated Erythrocytes                      | 49 |
| (c) Protective Effect of Glycerine and                   |    |
| Gelatin on Tannic acid-Old Tuber-                        |    |
| culin-treated Erythrocytes -----                         | 53 |



TABLE OF CONTENTS (continued)

|                                                                                                                    | Page |
|--------------------------------------------------------------------------------------------------------------------|------|
| (d) Protective Effect of N.R.S.<br>Absorbed with Sensitized Erythro-<br>cytes -----                                | 56   |
| (e) Protective Effect of N.R.S.<br>Absorbed with B.C.G. -----                                                      | 60   |
| (f) Effect of Homologous Serum on Sheep<br>Erythrocytes during Storage -----                                       | 64   |
| (g) Protective Effect of Bovine Albumin<br>Solution on Tannic acid-Old Tuber-<br>culin-treated Erythrocytes -----  | 67   |
| (h) Optimum Amounts of Tannic Acid and<br>Old Tuberculin for the Sensitiza-<br>tion of Sheep Erythrocytes -----    | 70   |
| 4. Application of the Above Findings to the<br>Examination of Experimental Rabbit Sera -                           | 74   |
| Absorption of Pre-inoculation Rabbit<br>Sera with Bovine Erythrocytes                                              |      |
| 5. Summary of the Technical Investigation<br>of Boyden's Modification of the Haem-<br>agglutination Reaction ----- | 82   |
| IV. <u>Clinical Trial of the Modified Boyden Haem-<br/>agglutination Technique</u>                                 |      |
| 1. Introduction -----                                                                                              | 83   |
| 2. Materials and Methods -----                                                                                     | 83   |
| 3. Results -----                                                                                                   | 84   |
| (a) Titration of Sera from Individuals<br>Suffering from Known Tuberculosis -                                      | 84   |
| Summary -----                                                                                                      | 90   |



TABLE OF CONTENTS (continued)

|                                                                                                              | Page |
|--------------------------------------------------------------------------------------------------------------|------|
| (b) Titration of Sera from Individuals<br>Not Suffering from Known Tuberculosis -----                        | 90   |
| (c) Titration of Sera from Individuals<br>with No Disease but with Known Exposure to Tubercle Antigens ----- | 93   |
| (d) Discussion of Results -----                                                                              | 99   |
| V. <u>Summary of Part II.</u> -----                                                                          | 100  |
| REFERENCES -----                                                                                             | 102  |
| APPENDIX                                                                                                     |      |
| I -----                                                                                                      | 111  |
| II -----                                                                                                     | 119  |
| III -----                                                                                                    | 120  |
| IV -----                                                                                                     | 121  |

# Table of Contents

|                      |    |
|----------------------|----|
| 1. Introduction      | 1  |
| 2. Methodology       | 2  |
| 3. Results           | 3  |
| 4. Discussion        | 4  |
| 5. Conclusion        | 5  |
| 6. References        | 6  |
| 7. Appendix          | 7  |
| 8. Glossary          | 8  |
| 9. Index             | 9  |
| 10. Bibliography     | 10 |
| 11. Acknowledgements | 11 |
| 12. Appendix A       | 12 |
| 13. Appendix B       | 13 |
| 14. Appendix C       | 14 |
| 15. Appendix D       | 15 |

THE SEROLOGICAL INVESTIGATION  
OF  
SYPHILIS AND TUBERCULOSIS

INTRODUCTION

The practice of sero-diagnosis in public health work is well established. In most instances, as in the investigation of gastrointestinal infections, the diagnosis is based on the agglutination of the infecting organism by the patient's serum. In other instances extracts of such organisms or suspensions of viruses or rickettsiae are employed as antigens in complement fixation techniques. In all these instances, however, the specificity of the tests is guaranteed by the specificity of the antigens employed. Curiously enough in two diseases, both of extreme importance to human welfare, comparable tests cannot be used because of the unsuitability or unavailability of the specific antigen. In syphilis and in tuberculosis, both chronic granulomatous diseases, we find this situation apparent. Nevertheless, in the first of these serological diagnosis of infection is among the best established of all serological procedures, while in the latter serological tests have regularly failed until recently to produce reputable results. In syphilis the established methods of sero-diagnosis depend on the use of a biologically non-specific antigen, non-specific in the sense that it contains absolutely no material related to the infecting organism; yet the sero-reaction with this non-specific antigen is universally recognized to be of very high clinical specificity. In tuberculosis, although the specific antigen is readily



available from cultures of infecting organisms, their physical nature apparently renders them unsuitable. Many attempts to develop serological tests have been based on the use of antigens derived from this specific material, but until recently the outstanding feature of these tests was the clinical non-specificity of the results.

From the pathological standpoint and even from the nature of clinical disease, syphilis and tuberculosis have much in common and the tests applied to their sero-diagnosis can legitimately be considered together. Yet the problems still associated with both these tests are entirely dissimilar. In syphilis we find that acceptable tests of considerable antiquity have been continually refined through the years until the technical details have reached a degree of standardization unusual in laboratory procedures. In most advanced communities surveys are regularly carried out to maintain the uniformity of findings and the clinical reliability of results. Yet the greatest measure of concern has always been with the techniques applied to individual tests and with the laboratory materials used in them. There still remains a very considerable problem in connection with the variable quality of the substance actually tested, namely human serum. This is intrinsically variable from person to person, a factor which is naturally quite uncontrollable, but in addition serum taken for such tests is handled or mishandled in many ways before it is finally subjected to the rigid control of the test proper. Variations will occur often to extreme degrees in the intervals between obtaining a blood sample and performing a test, in the temperatures to which such samples are exposed during that interval, and in the degree of agitation and even of extraneous bacterial contamination to which they are subjected. In tuberculosis only a recently elaborated sero-diagnostic procedure is worthy of consideration. Owing to its recent development few details of technique



have been standardized in a universally acceptable manner. Yet the reports of various workers are in general of sufficient promise to warrant further investigation of these technical procedures with the hope of increasing the clinical reliability of the test.

In this thesis it is proposed to investigate some of these problems; in the sero-diagnosis of syphilis to consider factors particularly applicable to the serum itself, in the sero-reaction for tuberculosis to concentrate on certain aspects of the technical performance of the tests. These will be considered in separate sections.



PART I

A Critical Review of The Sero-Diagnosis of Syphilis

Together with Some New Studies

Concerning The Stability of The Syphilitic Antibody



## SERO-DIAGNOSIS OF SYPHILIS

### I. HISTORICAL REVIEW

In 1901 Bordet and Gengou (1) published their observations of the phenomenon of complement fixation. They established the fact that complement is "fixed" by a combination of antibody plus antigen, but by neither alone. According to Kolmer (2), they failed at first to see the potentiality of this reaction as a diagnostic measure. However, other workers did so. Widal and Le Sourd (3) were among the first to apply it to the sera from typhoid patients, with Bact. typhosum as the antigen. When Schaudinn and Hoffmann (4) in 1905 discovered that Treponema pallidum was the causal agent of syphilis, it was inevitable that attempts would be made to apply sero-diagnostic techniques to this disease.

Within a short while, Wassermann, Neisser and Bruck (5) published their studies on the complement fixation reaction in syphilis using aqueous extracts of syphilitic tissues as antigens. They believed the reaction to be biologically specific.

This concept of the specificity of the "Wassermann reaction" was changed when Marie and Levaditi in 1907 (6) showed that a positive reaction occurred with syphilitic sera when an extract of normal tissues, such as guinea pig spleen, was used as antigen. Although this finding was disconcerting it was soon discovered that the test was still highly specific clinically. Thus the complement fixation test has become accepted for the diagnosis of syphilis on an empirical rather than biological basis. Today the antigens in general use are alcoholic extracts of beef heart muscle enhanced with cholesterol and lecithin (7).



Attempts of workers such as Beck (8), Erickson and Eagle (9) and Eagle and Hogan (10) to use antigens derived from various treponemes, theoretically more specific, have never led to generally accepted procedures, and have been criticized by Kolmer, Kast and Lynch (11). Thus, until the newer techniques of Nelson and Mayer (12) can be simplified to a point of reasonable applicability, tests of the Wassermann type will continue as one of the main diagnostic procedures in the control of syphilis.

Since its first appearance there have been numerous modifications of the original Wassermann reaction. In America the most frequently used procedures are those originated by Kolmer (13) and Eagle (14). In Great Britain the most favoured one appears to be the modification of Harrison-Wyler (15).

In addition to these complement fixation tests flocculation reactions are widely used. As early as 1907 Michaelis observed the phenomenon of flocculation when he added aqueous liver extract to a syphilitic serum. According to Hinrichsen (16) Meinicke published his technique for a flocculation test in 1917. This was later followed by the well-known modification of Sachs and Georgi.

The earlier flocculation tests required a prolonged incubation period. This difficulty was finally overcome when Kahn (17), who had studied under Meinicke, Sachs and Georgi, published the results of his studies in 1922. Since the introduction of his first diagnostic precipitation test Kahn has evolved a quantitative and a presumptive test. This latter test is not a diagnostic but a "screening" procedure using a "sensitized" antigen. Sera that give a positive result in the presumptive and diagnostic tests are usually tested quantitatively to determine the degree of reaction, and may even be submitted to a "verification test" (18) which, Kahn believes, differentiates the "false positive" from the specifically positive reaction.



Flocculation tests have also been devised by Kline and Young (19), Hinton (20), Eagle (21) and many others.

## II. EVALUATION AND STANDARDIZATION OF

### SERO-DIAGNOSTIC METHODS

It is clearly desirable to establish throughout the world uniform standards of specificity and sensitivity in the sero-diagnosis of syphilis. With this object, the Health Organization of the League of Nations sponsored the first serologic laboratory conference at Copenhagen in 1923. This was followed by the second Copenhagen conference in 1928 and a third in Montevideo in 1930 (22).

The second Copenhagen conference advocated the use of at least two different serologic tests for the serum diagnosis of syphilis. One of these was to be the Wassermann reaction and the second a flocculation test. Conforming with this policy the custom today is to perform at least two tests, one being a complement fixation reaction and the other a flocculation or precipitation reaction.

In the United States evaluation studies have been conducted since 1934. In the 1942 survey (23), each of the one hundred and fifteen participating laboratories tested approximately 230 syphilitic and 125 non-syphilitic sera by various sero-diagnostic methods. The range of sensitivity was between 60% and 95% with an average of 70% to 80%, and that of specificity was from 86% to 100%, with an average of 95% to 100%. For example, the Kahn Standard Precipitation test was performed by 34 laboratories with 8020 syphilitic and 4396 non-syphilitic sera, sensitivity of 62.6% to 87.0% and specificity of 96.7% to 100% were obtained. In the same study, the Kolmer Simplified Complement Fixation test was carried out by



16 laboratories with 3698 syphilitic and 2024 non-syphilitic sera, the sensitivity varied from 70.8% to 88.5% and the specificity from 99.2% to 100%.

Of particular interest are the evaluation studies in Canada which are conducted every two years. The Laboratory of Hygiene in Ottawa distributes the test sera to the provincial testing centres. In the 1952-1953 survey (Appendix I) the four most frequently used serological methods were performed in 5 to 10 laboratories. The specificity of all methods was practically 100%. The sensitivity shown is lower than in the United States because it is evaluated on the basis of the clinical diagnoses of the individuals from whom the sera were obtained. More important is the demonstration that the results from the individual Provincial Laboratories are in close accord with each other and with the results of the central serological laboratory in Ottawa.

### III. LIMITATIONS OF THE EVALUATION STUDIES

It is clearly evident that the sera chosen for trial in these studies are not identical by the time they are tested in the various centres to which they have been distributed from the common source. The state of the serum may be affected by a number of variable factors, such as the time required for the specimen to reach its destination, the changes in temperature and the manipulation to which it may be subjected during transport. The use of stable lyophilized sera would to some extent overcome these difficulties. The attainment of greater specificity and sensitivity might then be possible, provided the participating laboratories adhered meticulously to the same technical procedures.



#### IV. EXPERIMENTAL

##### The Influence of Some Physical Factors on the Stability of Normal and Syphilitic Sera



## A. The Influence of Age and Temperature on Sera

### Introduction

Every central laboratory receives blood specimens from a wide area and many specimens may have to be transported hundreds of miles. Consequently several days may elapse before the tests are performed. Some of these samples of blood and also of cerebro-spinal fluid exhibit anti-complementary properties, that is, the fluids--serum or C.S.F.--possess the ability to remove or deviate complement in the absence of antigen when they are tested. The incidence of this property in sera and cerebrospinal fluids at the Provincial Laboratory of Public Health, Edmonton (24) is shown in Table I. A total of 5276 sera and 1922 cerebrospinal fluids was examined by the Kolmer Simplified and Quantitative Complement Fixation tests, respectively. In this series, 1.29% of the sera and 0.53% of the cerebrospinal fluids were found to be anticomplementary.

TABLE I

### Occurrence of Anticomplementary Sera and C.S.F.

as shown by the Kolmer Complement Fixation Reaction, 1953

| <u>Result</u>     | <u>Sera</u> |        | <u>C.S.F.</u> |        |
|-------------------|-------------|--------|---------------|--------|
|                   | No.         | %      | No.           | %      |
| Negative          | 3672        | 69.90  | 1863          | 96.93  |
| Positive          | 1536        | 29.11  | 49            | 2.54   |
| Anticomplementary | 68          | 1.29   | 10            | 0.53   |
| Total             | 5276        | 100.00 | 1922          | 100.00 |



Eagle (25) detected no change in normal sterile sera stored for one, two and four months in the icebox. On the other hand, syphilitic serum tends to lose its reagin content even at refrigerator temperature whether left on, or removed from the clot. This is shown by the data in Table II (24).



TABLE II

Effect of Storage on Syphilitic Sera Held at 2-4°C.  
as Reflected by the Results of the Standard Kahn Test



TABLE II

| Serum No.                             | Serum removed from the clot - Time in days |        |         |        |         |         |         |        |         |        |         |        |         |        |
|---------------------------------------|--------------------------------------------|--------|---------|--------|---------|---------|---------|--------|---------|--------|---------|--------|---------|--------|
|                                       | 1st                                        |        | 2nd     |        | 3rd     |         | 4th     |        | 5th     |        | 7th     |        | 9th     |        |
|                                       | Reading                                    | Result | Reading | Result | Reading | Result  | Reading | Result | Reading | Result | Reading | Result | Reading | Result |
| 1                                     | 122                                        | Dbt.★  | 222     | Dbt.   | 222     | Dbt.★★  | ---     | ---    | ---     | ---    | ---     | ---    | ---     | ---    |
| 2                                     | 012                                        | "      | 001     | Neg.   | -01     | Neg.★★★ | ---     | ---    | ---     | ---    | ---     | ---    | ---     | ---    |
| 3                                     | 233 <sup>¢</sup>                           | "      | ---     | ¢¢     | 022     | Dbt.    | ---     | ---    | 022     | Dbt.   | ---     | ---    | ---     | ---    |
| 4                                     | 133                                        | "      | ---     | ---    | 012     | "       | ---     | ---    | 012     | "      | ---     | ---    | ---     | ---    |
| 5                                     | 222                                        | "      | ---     | ---    | 012     | "       | ---     | ---    | 012     | "      | ---     | ---    | ---     | ---    |
| 6                                     | 431                                        | Pos.   | ---     | ---    | 432     | Pos.★★  | ---     | ---    | 432     | Pos.   | ---     | ---    | ---     | ---    |
| 7                                     | 233                                        | Pos.   | ---     | ---    | 133     | Dbt.    | ---     | ---    | -22     | Dbt.   | ---     | ---    | ---     | ---    |
| 8                                     | 222                                        | Dbt.   | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 222     | Dbt.   | -22     | Dbt.   |
| 9                                     | 233                                        | "      | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 023     | "      | 022     | "      |
| 10                                    | 123                                        | "      | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | ---     | ---    | ---     | ---    |
| 11                                    | 422                                        | Pos.   | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 322     | "      | -22     | "      |
| 12                                    | 133                                        | Dbt.   | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 022     | "      | 022     | "      |
| 13                                    | 022                                        | "      | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 011     | Neg.   | 011     | Neg.   |
| 14                                    | 012                                        | "      | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | ---     | ---    | ---     | ---    |
| 15                                    | 432                                        | Pos.   | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 311     | Dbt.   | 110     | "      |
| 16                                    | 333                                        | "      | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 222     | "      | 222     | Dbt.   |
| 17                                    | 122                                        | Dbt.   | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | 0+1     | Neg.   | 000     | Neg.   |
| 18                                    | 012                                        | "      | ---     | ---    | ---     | ---     | ---     | ---    | ---     | ---    | ---     | ---    | ---     | ---    |
| Serum left on the clot - Time in days |                                            |        |         |        |         |         |         |        |         |        |         |        |         |        |
| 15                                    | 222                                        | Dbt.   | ---     | ---    | ---     | ---     | 122     | Dbt.   | ---     | ---    | 122     | Dbt.   | ---     | ---    |
| 16                                    | 422                                        | Pos.   | ---     | ---    | ---     | ---     | 432     | Pos.   | ---     | ---    | 321     | "      | ---     | ---    |
| 17                                    | 444                                        | "      | ---     | ---    | ---     | ---     | 444     | "      | ---     | ---    | 210     | "      | ---     | ---    |
| 18                                    | 122                                        | Dbt.   | ---     | ---    | ---     | ---     | 011     | Neg.   | ---     | ---    | 001     | Neg.   | ---     | ---    |
| 19                                    | 344                                        | Pos.   | ---     | ---    | ---     | ---     | 344     | Pos.   | ---     | ---    | 344     | Pos.   | ---     | ---    |
| 20                                    | 444                                        | Pos.   | ---     | ---    | ---     | ---     | 444     | Pos.   | ---     | ---    | 210     | Dbt.   | ---     | ---    |

★ = Doubtful

★★ = Positive

★★★ = Negative

¢ = Upper row of figures is the first reading, second row, the second. Where only one row is shown, the two readings are identical.

¢¢ = Not done



It was of interest, therefore, to determine what effect storage of normal sera at 37°C. and 2-4°C., and of syphilitic sera at 37°C. would have on the outcome of the serologic test.

### Materials and Method

The syphilitic and non-syphilitic sera for this experiment were generously supplied by the Provincial Laboratory of Public Health, Edmonton. All sera had been heated at 56°C. for 30 minutes. The tubes with the sera were stoppered with sterile corks, one series of 11 normal sera was held at 37°C. for 21 days, a second series of 20 normal sera was stored at 2-4°C. for 5 and 12 days, a third group of 11 syphilitic sera was stored at 37°C. for 5 days. These tubes were cotton-plugged. At the end of incubation, the serum samples were heated for 10 minutes and tested by the Kolmer Simplified Complement Fixation method.

### Results

The results of the Kolmer test of the normal sera are shown in Table III (a). No changes were demonstrable in any group of normal sera.

TABLE III (a)

Results of the Kolmer Complement Fixation Test  
of Sera stored at 37°C. and 2-4°C.

| No. of Sera | Temp. of Incubation<br>°C. | Time in Days |    |    | Anticomplementary Property |
|-------------|----------------------------|--------------|----|----|----------------------------|
|             |                            | 5            | 12 | 21 |                            |
| 20          | 2-4                        | 0            | 0  | -  | 0                          |
| 11          | 37                         | -            | -  | 0  | 0                          |



The results of the serological tests of the syphilitic sera before and after storage are shown in Table III (b). When the amount of serum was insufficient for the complement fixation test and for the serum control, the latter was done alone to observe any anticomplementary activity. The small amounts of serum available at the end of the period of storage allowed only one serological test to be performed. It was found that of the 9 sera retested, 3 were unaffected by storage, 5 became anticomplementary and one changed from a negative to a doubtful complement fixation. Two sera evaporated in the incubator.



TABLE III (b)

†

Results of Serological Tests of Syphilitic Sera  
before and after holding at 37°C. for 5 days



TABLE III (b)

| Serum No. | Results before holding |                  |                   |               | Kolmer C.F.T. after holding |         |                       |
|-----------|------------------------|------------------|-------------------|---------------|-----------------------------|---------|-----------------------|
|           | Presump. Kahn          | Standard Kahn    | Quantitative Kahn | Kolmer C.F.T. | Test                        | Control | Change                |
| 8         | 3+                     | 432              | 2444332100        | ++++          | --                          | --      | --                    |
| 10        | 2+                     | 023              | --                | ++++          | ++++                        | 0       | none                  |
| 12        | 3+                     | 432              | 044321            | ++++          | ++++                        | +       | sl. ac. <sup>★★</sup> |
| 19        | 2+                     | 012              | --                | ++++          | ++++                        | +       | " "                   |
| 29        | 3+                     | 334              | 432000            | ++++          | --                          | --      | --                    |
| 43        | 4+                     | 233              | 200000            | ++++          | +++                         | ++      | ac.                   |
| 72        | 2+                     | 122              | --                | ++++          | ++++                        | 0       | none                  |
| 109       | 3+                     | 112              | --                | 0             | dbt. <sup>***</sup>         | 0       | 0 to dbt.             |
| 116       | 4+                     | 334              | 219000            | ++++          | ++++                        | +       | sl. ac.               |
| 125       | 2+                     | 122 <sup>*</sup> | --                | ++++          | --                          | +       | " "                   |
|           |                        | 012              |                   |               |                             |         |                       |
|           |                        | 123              |                   |               |                             |         |                       |
| 197       | 4+                     | 023              | --                | ++++          | ++++                        | 0       | none                  |

\* Upper row of figures = 1st reading.  
 Lower row of figures = 2nd reading.  
 Where only one row, both readings were identical.

★★ Slightly anticomplementary.

\*\*\* Doubtful.



## Conclusion

Normal sera as shown in this experiment were stable for considerable periods of time, even at the extreme temperature of 37°C. The lability of the syphilitic sera was found to be emphasized at 37°C. At 2-4°C. such sera have been found to lose their reactivity in 5-9 days (Table II). At 37°C. the majority of the syphilitic sera in this study exhibited the anticomplementary property in 5 days.

## B. The Influence of Bacteria on the Anticomplementary Activity of Serum

## Introduction

From the above observations the anticomplementary action of sera and C.S. fluids cannot be ascribed solely to the effects of ageing and temperature. This is confirmed by the fact that 51 of the 65 anticomplementary sera and 6 of the 10 anticomplementary C.S. fluids (Table I) were received from local urban centres, the rest from rural areas. A possible factor of importance is the degree of bacterial contamination of such materials (26), a factor which is to some degree independent of the point of origin of the specimen. This was investigated experimentally. Known anticomplementary sera were cultured to find if bacteria were present and normal sera contaminated with bacteria to determine if this induced an anticomplementary effect.

## Materials and Methods

Thirteen sera, found to be anticomplementary in routine serological tests, were examined. From five of these only samples previously inactivated at 56°C. were available, but from eight unheated aliquots were obtained. Each serum was cultured by adding one drop of the



specimen to a tube of serum broth. The cultures were incubated for 24 hours at 37°C. Only two sera, both previously inactivated, showed growth in the serum broth, in one the organisms were gram-positive bacilli, and in the second, of which inactivated and unheated samples were available, the organisms in both samples were gram-positive cocci and bacilli. The organisms were not identified.

A series of 150 sera negative by the Kahn Presumptive test, of which 145 had been inactivated, was first tested for sterility by inoculating one drop of each serum into a tube of serum broth. Only four of these showed any growth. Two cultures (of inactivated sera) consisted of gram-positive cocci, one (of inactivated serum) of gram-negative bacilli and one (of unheated serum) of gram-positive bacilli. The organisms were not identified. Each of the 150 specimens, including the four contaminated sera, was then divided into two portions. In 17 specimens one aliquot of 1.0 ml. was inoculated with one loopful of a 24 hour broth culture of Staphylococcus aureus and in 133 specimens one aliquot of 1.0 to 2.0 ml. was inoculated similarly with Bacillus subtilis. The second aliquot of approximately 1.0 ml. was always retained as a control. All tubes were capped with bakelite screw tops and incubated for periods of 3, 5-6, 9-10 and 13-15 days at 37°C. Growth of the organisms was recorded in plus signs. Prior to carrying out the Kolmer Simplified Complement Fixation test, the serum specimens were centrifuged for 15 minutes at 2000 r.p.m. The supernates were inactivated for 30 minutes at 56°C. in the case of the unheated sera and for 10 minutes in the case of the previously heated ones. The degree of deviation of complement was denoted by plus signs.

### Results

All the specimens contaminated with S. aureus were markedly



anticomplementary after 5-6 days of incubation. One of the two naturally contaminated sera in this series was seen to be slightly anticomplementary in the control test while the second was found to be equally anticomplementary in the artificially contaminated portion and in the control.

In the B. subtilis series a number of changes were recorded. In the 3 day group only one of 20 sera became anticomplementary; in the 5-6 day series 41 (40.6%) became anticomplementary and 51 (51.5%) were unchanged; the remaining 8 (7.9%) showed a most interesting phenomenon. No anticomplementary effect developed but the serological test result changed from negative to positive. No such change was observed in the control sera of these specimens though 2 became anticomplementary. However, no significance can be ascribed to this observation because 3 sterile controls belonging to other sera which became anticomplementary gave a final test reading which could have been interpreted as positive.

In the 9-10 day and 13-15 day groups 7 (46.7%) and 15 (41.7%), respectively, developed the anticomplementary effect. In the latter group of sera 7 were so heavily contaminated as to be unsuitable for the test. It was of interest to note that the remaining two originally naturally contaminated sera were unchanged in the control portions and anticomplementary in the artificially contaminated aliquots.

Table IV shows the results of 20 sera representative of this series.



TABLE IV

Kolmer Simplified Complement Fixation Test  
of Sera Inoculated with B. subtilis after Test for Sterility,  
and Held at 37°C. for 3, 5-6, 9-10, and 13-15 Days



TABLE IV

| No. of Serum         | Growth of Serum in Serum Broth | Growth of B. subtilis in Serum | Incubation at 37°C. in days |         |               |         |                     |         |               |         |
|----------------------|--------------------------------|--------------------------------|-----------------------------|---------|---------------|---------|---------------------|---------|---------------|---------|
|                      |                                |                                | C.F.T. in 3 days            |         |               |         | C.F.T. in 9-10 days |         |               |         |
|                      |                                |                                | Inoculated Serum            |         | Control Serum |         | Inoculated Serum    |         | Control Serum |         |
|                      |                                |                                | Test                        | Control | Test          | Control | Test                | Control | Test          | Control |
| 3                    | 0                              | ++                             | 0                           | 0       | --            | --      | 0                   | 0       | 0             | 0       |
| 4                    | 0                              | ++                             | 0                           | 0       | --            | --      | +++                 | ++      | --            | --      |
| 5                    | 0                              | ++                             | 0                           | 0       | --            | --      | 0                   | 0       | 0             | 0       |
| 11                   | 0                              | +                              | 0                           | 0       | --            | --      | ++                  | ++      | --            | --      |
| 123                  | 0                              | +                              | 0                           | 0       | --            | --      | +++                 | ++      | 0             | 0       |
| 124                  | 0                              | +++                            | ++++                        | ++++    | --            | --      | --                  | --      | --            | --      |
| C.F.T. in 5-6 days   |                                |                                |                             |         |               |         |                     |         |               |         |
| 74                   | 0                              | ++                             | 0                           | 0       | 0             | 0       | 0                   | 0       | --            | --      |
| 88                   | 0                              | ++                             | +                           | 0       | 0             | 0       | ++++                | ++      | --            | --      |
| 104                  | 0                              | ++                             | 0                           | 0       | 0             | 0       | ++++                | ++      | --            | --      |
| 190                  | 0                              | +++                            | 0                           | 0       | 0             | 0       | ++++                | +++     | 0             | 0       |
| 27                   | 0                              | +                              | 0                           | 0       | 0             | 0       | --                  | ++++    | --            | --      |
| 39                   | 0                              | +                              | 0                           | 0       | 0             | 0       | 0                   | 0       | --            | --      |
| 36                   | 0                              | ++++                           | +++                         | ++++    | --            | --      | --                  | --      | --            | --      |
| 44                   | 0                              | ++++                           | ++                          | ++      | 0             | 0       | --                  | --      | --            | --      |
| 45                   | 0                              | ++++                           | 0                           | 0       | 0             | 0       | +                   | +       | --            | --      |
| 55-1                 | +<br>Gram-pos.<br>cocci        | ++++                           | +++                         | ++      | 0             | 0       | --                  | --      | --            | --      |
| 51                   | 0                              | ++                             | 0                           | 0       | 0             | 0       | ++++                | ++++    | --            | --      |
| 55-2                 | 0                              | ++                             | 0                           | 0       | 0             | 0       | 0                   | 0       | --            | --      |
| 56                   | 0                              | ++++                           | ++++                        | ++++    | 0             | 0       | --                  | --      | --            | --      |
| 9                    | 0                              | +                              | 0                           | 0       | --            | 0       | --                  | ++++    | --            | --      |
| C.F.T. in 13-15 days |                                |                                |                             |         |               |         |                     |         |               |         |
| 74                   | 0                              | ++                             | 0                           | 0       | 0             | 0       | 0                   | 0       | --            | --      |
| 88                   | 0                              | ++                             | +                           | 0       | 0             | 0       | ++++                | ++      | --            | --      |
| 104                  | 0                              | ++                             | 0                           | 0       | 0             | 0       | ++++                | ++      | --            | --      |
| 190                  | 0                              | +++                            | 0                           | 0       | 0             | 0       | ++++                | +++     | 0             | 0       |
| 27                   | 0                              | +                              | 0                           | 0       | 0             | 0       | --                  | ++++    | --            | --      |
| 39                   | 0                              | +                              | 0                           | 0       | 0             | 0       | 0                   | 0       | --            | --      |
| 36                   | 0                              | ++++                           | +++                         | ++++    | --            | --      | --                  | --      | --            | --      |
| 44                   | 0                              | ++++                           | ++                          | ++      | 0             | 0       | --                  | --      | --            | --      |
| 45                   | 0                              | ++++                           | 0                           | 0       | 0             | 0       | +                   | +       | --            | --      |
| 55-1                 | +<br>Gram-pos.<br>cocci        | ++++                           | +++                         | ++      | 0             | 0       | --                  | --      | --            | --      |
| 51                   | 0                              | ++                             | 0                           | 0       | 0             | 0       | ++++                | ++++    | --            | --      |
| 55-2                 | 0                              | ++                             | 0                           | 0       | 0             | 0       | 0                   | 0       | --            | --      |
| 56                   | 0                              | ++++                           | ++++                        | ++++    | 0             | 0       | --                  | --      | --            | --      |
| 9                    | 0                              | +                              | 0                           | 0       | --            | 0       | --                  | ++++    | --            | --      |

C.F.T. = Complement fixation test.

"--" = Not done.

"0" = Negative.



In Table V the changes in both the S. aureus and the B. subtilis series of sera, as a result of the presence of bacteria and/or their products, are shown.

Due to evaporation not all sera were tested after each incubation period.

5 sera were tested after 3 days

15 " " " " 3 and 9-10 days

94 " " " " 5-6 days

24 " " " " 5-6 and 13-15 days

5 " " " " 13-15 days

7 sera were unfit for test.

150

In general it was noticed that the heavier the growth of the contaminant the more likely were anticomplementary effects to develop. For 38 sera of the 5-6 day group, the bacterial growth was not recorded, in the remaining 80 sera the development of anticomplementary activity seemed to be directly related to the heaviness of growth; it occurred in: -

31% of sera with a 4+ growth of B. subtilis

4% " " " " 3+ " " " "

5% " " " " 2+ " " " "

3% " " " " 1+ " " " "

while 57% of the sera remained unaffected.



TABLE V

Changes Produced in Sera

Due to Bacterial Contamination, Age and Temperature

as Indicated by the Kolmer Simplified Complement Fixation Test



TABLE V

| Incuba-<br>tion in<br>days | Contaminated Sera |                |       |                                   | Control Sera<br>Changes observed |                 |      |               |
|----------------------------|-------------------|----------------|-------|-----------------------------------|----------------------------------|-----------------|------|---------------|
|                            | Organism<br>used  | No.            | %     | Changes<br>observed               | ac. <del>ant</del>               | Neg. to<br>Pos. | None | N.S.Q. $\phi$ |
| 3                          | B. subtilis       | 19             | 95.00 | none                              | -- $\phi\phi$                    | --              | --   | 19            |
|                            | "                 | 1              | 5.00  | ac.                               | --                               | --              | --   | 1             |
| 5-6                        | S. aureus         | 5 <sup>★</sup> | 29.4  | ac.                               | 1                                | --              | --   | 4             |
|                            | "                 | 12             | 70.6  | ac.                               | 1                                | --              | 11   | --            |
|                            | B. subtilis       | 41             | 40.6  | ac.                               | --                               | 3               | 19   | 19            |
|                            | "                 | 51             | 51.5  | none                              | --                               | --              | 33   | 19            |
|                            | "                 | 8              | 7.9   | Neg. to Pos.                      | 2                                | --              | 4    | 2             |
| 9-10                       | B. subtilis       | 7              | 46.7  | ac.                               | --                               | --              | 1    | 6             |
|                            | "                 | 8              | 53.3  | none                              | --                               | --              | 3    | 5             |
| 13-15                      | B. subtilis       | 7              | 19.4  | unfit. for<br>test <del>ant</del> | --                               | --              | --   | --            |
|                            | "                 | 15             | 41.7  | ac.                               | --                               | --              | 1    | 14            |
|                            | "                 | 14             | 38.9  | none                              | --                               | --              | --   | 14            |

★ = unheated sera, all the rest had been inactivated.

~~ant~~ = anticomplementary activity.

~~ant~~ = bacterial growth extremely heavy.

$\phi$  = insufficient for test.

$\phi\phi$  = change not shown.



## Conclusion

Little evidence was obtained on the importance of bacterial contamination on the incidence of natural anticomplementary activity. Only a small number of sera was available and of these nearly half had been inactivated. Certainly general laboratory experience suggests that inactivation is not an effective sterilizing procedure for serum, a fact borne out by observations in the current series, but it cannot be disregarded in argument. From the recorded observations, however, no evidence has been obtained that bacterial contamination is a significant factor in naturally occurring anticomplementary sera.

The second part of this experiment showed that artificial contamination of normal sera with bacteria did exercise some influence. The combination of ageing, exposure to incubator temperature and bacterial contamination led to the development of anticomplementary activity in a high percentage of sera in certain experiments. The physical presence of bacteria itself ~~may~~<sup>may</sup> be a responsible factor because contamination with both S. aureus and B. subtilis was effective in the development of anticomplementary activity, although not to the same extent. It is possible, therefore, that some bacteria are more effective in inducing this phenomenon than others and even to suggest that this may occasionally be of importance in naturally occurring anticomplementary activity.

These findings are in agreement with those of Kendrick (27) who used S. aureus and a gram-negative bacillus isolated from a naturally contaminated serum. Negative and positive, unheated and heated sera were contaminated. Kendrick found that changes occurred more frequently in the unheated negative and positive groups of sera than in the heated specimens.

Incidentally, the change in serological reaction observed in



certain of the investigated sera emphasizes the desirability of increased knowledge of all factors which may influence such specimens submitted for routine tests.

### C. Stability of Lyophilized Syphilitic Sera of Known Reactivity

#### Introduction

The lack of "stability" of syphilitic sera has been demonstrated in the previous sections, yet to control tests in sensitivity and specificity, either on a national scale or in individual laboratories, such stability is essential. Nowadays the problem is rendered more crucial by the difficulty of finding untreated cases with high "reagin" titres, and by the rapid disappearance of such titres under modern specific treatment. Surprisingly little work on the matter has been reported. Harris, Harding and Bossak (28) describe a successful method of preserving dehydrated serum for longer than a year at refrigerator temperature. Cannefax (29) reports on a processed serum stable for at least 15 months in the dried state and for fifty days after being reconstituted if the serum is kept frozen. Flo sdorf and Mudd (30), the originators of the "lyophile" and "cryochem" methods of freeze-drying biological materials, report the stability of such substances as complement serum, human milk and convalescent serum. An attempt to preserve some syphilitic sera by lyophilization is reported in this study.

#### Materials and Methods

The syphilitic sera for this work were obtained through the kindness of the Division of Social Hygiene, Provincial Clinic, Edmonton.



Sera were obtained from patients diagnosed as syphilitic either on dark-field examination or on clinical and serological findings. The difficulty of obtaining suitable samples from a routine clinic was emphasized by the fact that of 24 specimens received only 5 were considered worth processing. The others were discarded because of feeble serological reactions, haemolysis or insufficient amount. As far as could be ascertained all but one of the five sera were from treated cases of syphilis. As soon as possible after receipt of the blood samples, the sera were removed from the clot. An aliquot was used for the serological tests. Where indicated by the Kahn Presumptive test results, the Kahn Standard and Quantitative and the Kolmer Simplified Complement Fixation tests were carried out. Sera with a positive 3 or 4 report by the Standard Kahn and with a 4-8 dilutions report by the Quantitative Kahn, and free from haemolysis, were chosen for preservation. The serum was dispensed in 0.25 or 0.5 ml. amounts into 1.0 ml. pyrex tubes, 10.6 x 0.7 cm., with constrictions 4 cm. from the open end. The tubes were loosely plugged with cotton. The material was freeze-dried in the Edwards Centrifugal Freeze Dryer, Model 2A/122 (Plate I) according to the technique of Fry and Greaves (31).

This apparatus consists of a vacuum chamber containing a small electric motor fitted with a centrifuge head which holds forty 1.0 ml. tubes. The head is surrounded by four ring-shaped trays for the phosphorous pentoxide which reacts with the water. A Pirani vacuum gauge indicates the pressure. The centrifuge is started, at a speed of 700 r.p.m. the vacuum pump is turned on to evacuate the chamber rapidly. In approximately 4 minutes the pressure should be 1 mm. Hg, at this point "snap-freezing" occurs. Frothing is counteracted by the centrifugal force. The centrifuge is run 5 minutes longer. The Pirani gauge should now register 0.01 mm. Hg or lower. This pressure is maintained for 4-6 hours, depending on the



load, to effect complete drying. After the drying period the vacuum is decreased slowly. When this operation is completed, the tubes are removed from the centrifuge, the cotton plugs are removed and the tubes are then attached securely to the vacuum manifold which is mounted on the dryer in a vertical position. A further evacuation is carried out for half an hour over  $P_2O_5$ , followed by checking the ampoules for vacuum tightness by passing a high frequency electrical discharge lightly over the surface of the tubes. If no leaks are evident the tubes are sealed with a cross-fire burner.

The ampoules of sera #2023 and #2088 (designated Group I) were stored at room temperature, those of sera #2552, #2555 and #2575 (Group II) were divided into 3 lots, one lot was held at room temperature, the second at  $37^{\circ}C$ . and the third at  $2-4^{\circ}C$ . Group I sera were tested after 6 and 29 weeks, those of Group II after 4 and 26 weeks. All dried sera except those tested after 6 weeks, were reconstituted with sterile distilled water. The exception (Group I, 6 weeks) was dissolved in sterile normal saline.

## Results

Considerable difficulty was experienced in dissolving the dehydrated material. Even after re-hydration such sera presented a cloudy, fuzzy appearance with visible particles. This necessitated centrifuging the specimens prior to testing.

The reconstituted sera were inactivated at  $56^{\circ}C$ . for 30 minutes. The Standard and Quantitative Kahn and the Kolmer Simplified Complement Fixation tests were carried out where the volume of serum permitted. The results are shown in Table VI, as well as the results of the initial pre-freeze-drying serological tests.



TABLE VI

Effect of Freeze-drying and Storage on Syphilitic Sera



TABLE VI

| Serologic Tests    |               |               |        |                      |                 | Storage of<br>Freeze-dried sera |                 | Notes                                                                                           |
|--------------------|---------------|---------------|--------|----------------------|-----------------|---------------------------------|-----------------|-------------------------------------------------------------------------------------------------|
| No.<br>of<br>Serum | Kahn<br>Pres. | Kahn Standard |        | Kahn<br>Quantitative | Kolmer<br>S.CFT | at<br>Temp.                     | for<br>Duration |                                                                                                 |
|                    |               | Reading       | Result |                      |                 |                                 |                 |                                                                                                 |
| 2023               | 4+            | 443           | Pos. 4 | 444000               | ++++            | Room                            | 6 wks.          | Initial tests<br>Reconstituted with<br>0.9% NaCl                                                |
| "                  | --            | --4           | Pos. 4 | 431000               | ++++            | "                               | 29 wks.         | Reconstituted with<br>distilled water<br>Total vol. of serum:<br>2.75 ml.                       |
| "                  | --            | 444           | Pos. 4 | 444000               | --              | "                               |                 |                                                                                                 |
| 2088               | 4+            | 344           | Pos. 4 | 430000               | ++              | Room                            | 6 wks.          | Initial tests                                                                                   |
| "                  | --            | --4           | Pos. 4 | 421000               | ++++★           | "                               | 29 wks.         | Reconstituted with 0.9% NaCl<br>Reconstituted with dist. water<br>Total vol. of serum:<br>3 ml. |
| "                  | --            | 444           | Pos. 4 | 431000               | --              | "                               |                 |                                                                                                 |
| 2555               | 4+            | 344           | Pos. 4 | 321000               | +++             | 2-4°C.                          | 4 wks.          | Initial tests                                                                                   |
| "                  | --            | 233           | Pos. 3 | 310000               | --              | "                               | "               | Unsealed ampoules                                                                               |
| "                  | --            | 344           | Pos. 4 | 310000               | --              | 37°C.                           | "               | Reconstituted with<br>distilled water                                                           |
| "                  | --            | 344           | Pos. 4 | 320000               | --              | Room                            | "               |                                                                                                 |
| "                  | --            | 344           | Pos. 4 | 310000               | --              | 2-4°C.                          | 26 wks.         | Total volume of serum:<br>7 ml.                                                                 |
| "                  | --            | 344           | Pos. 4 | 430000               | --              | "                               | "               |                                                                                                 |
| "                  | --            | 244           | Pos. 4 | --                   | --              | 37°C.                           | "               |                                                                                                 |
| "                  | --            | 444           | Pos. 4 | --                   | --              | Room                            | "               |                                                                                                 |
| "                  | --            | 344           | Pos. 4 | 320000               | --              | "                               |                 |                                                                                                 |
| "                  | --            | 244           | Pos. 4 | --                   | --              | "                               |                 |                                                                                                 |
| 2552               | 3+            | 444           | Pos. 4 | 312000               | ++++            | 37°C.                           | 4 wks.          | Initial tests                                                                                   |
| "                  | --            | 443           | --     | 210000               | --              | Room                            | "               | Total volume of serum:<br>3.5 ml.                                                               |
| "                  | --            | 432           | Pos. 4 | 320000               | --              | "                               | "               |                                                                                                 |
| "                  | --            | 431           | Pos. 4 | 332000               | --              | 2-4°C.                          | 26 wks.         |                                                                                                 |
| "                  | --            | 443           | Pos. 4 | --                   | --              | 37°C.                           | "               |                                                                                                 |
| "                  | --            | 442           | Dbt. + | --                   | --              | Room                            | "               |                                                                                                 |
| "                  | --            | -32           | Pos. 3 | 322000               | --              | "                               | "               |                                                                                                 |
| "                  | --            | -31           | Pos. 3 | --                   | --              | "                               | "               |                                                                                                 |
| "                  | --            | 432           | Pos. 3 | --                   | --              | "                               | "               |                                                                                                 |
| "                  | --            | 431           | Pos. 3 | --                   | --              | "                               | "               |                                                                                                 |
| 2575               | 4+            | 344           | Pos. 4 | 421000               | 0               | 37°C.                           | 4 wks.          | Initial tests                                                                                   |
| "                  | --            | 002           | Neg.   | 100000               | --              | Room                            | "               | Total volume of serum:<br>2.5 ml.                                                               |
| "                  | --            | 002           | Neg.   | 100000               | --              | "                               | "               | N.S.Q. <del>44</del>                                                                            |
| "                  | --            | 001           | --     | --                   | --              | 2-4°C.                          | "               |                                                                                                 |
| "                  | --            | --            | Dbt. + | --                   | --              | 37°C.                           | 26 wks.         |                                                                                                 |
| "                  | --            | -00           | Dbt. + | --                   | --              | Room                            | "               |                                                                                                 |
| "                  | --            | -32           | Neg.   | 200000               | --              | "                               | "               |                                                                                                 |
| "                  | --            | 000           | Neg.   | --                   | --              | "                               | "               |                                                                                                 |
| "                  | --            | 010           | Neg.   | --                   | --              | "                               | "               |                                                                                                 |

★ = slightly anticomplementary.

★★ = Not sufficient quantity to be stored at 2-4°C.

"0" = negative.

"--" = not done.



It is seen that Group I sera remained unchanged, except that serum #2088 became slightly anticomplementary as shown by the Kolmer test at the end of 6 weeks. Lack of serum made further tests impossible. Two of the three sera of Group II showed some loss of potency, particularly serum #2575. This serum was not stored at 2-4°C. Serum #2555 remained stable for as long as tests were carried out. Two ampoules of this specimen of serum showed leaks and were stored in the refrigerator. After 4 weeks this portion of the serum showed a slightly lower reading than the same serum in sealed ampoules under identical conditions. The third serum #2552 was affected more by storage at 37°C. than at the other two temperatures.

### Conclusions

The results of the above experiments were unsatisfactory chiefly because it was impossible to obtain sufficient quantities of suitable sera. This, of course, re-emphasizes the urgency of the whole problem. Certain tentative conclusions, however, can be drawn. It is possible to preserve certain syphilitic sera by lyophilization. It is probable that storage under vacuum and at low temperature is most satisfactory. Samples which appear stable after initial tests at 4-6 weeks are likely to remain so for a considerable period. The difficulty of suitable reconstitution has already been mentioned and obviously necessitates further investigation.



Summary of Part I, Sections A, B and C.

It has been shown in this study that age and temperature within reasonable natural limits did not affect the stability of normal sera for considerable periods of time. Syphilitic sera on the other hand were found to develop the anticomplementary property to a marked extent at 37°C.

In naturally occurring anticomplementary sera, the role of bacteria cannot be said to be of great consequence on the basis of the small number of such sera that were examined. The possible importance of bacterial contamination, however, cannot be disregarded because artificial contamination together with age and exposure to incubator temperature (37°C.) exerted considerable influence on the serological reactions. A high percentage of sera became anticomplementary and a small number of sera changed from a negative to a positive serological reaction. A similar change from negative to positive reactions was observed in 3 specimens which served as serum controls.

Dehydrated syphilitic sera, the product of lyophilization, proved to be difficult to reconstitute satisfactorily, but even so, such reconstituted specimens were shown to retain their reactivity for periods up to 29 weeks, especially when the freeze-dried material was sealed in vacuo and stored at low temperatures.



PART II

Studies of The Haemagglutination Reaction

in

Tuberculosis



## SERO-DIAGNOSIS OF TUBERCULOSIS

### I. HISTORICAL REVIEW

The sero-diagnosis of tuberculosis was studied by Wassermann and Sachs (32), Wassermann and Bruck (33), Calmette and Massol (34), Bronfenbrenner (35), Wadworth, Maltaner and Maltaner (36), and Klopstock (37). These workers considered the test of limited diagnostic value mainly because an antigen of uniform reactivity could not be prepared.

The antigens used were of three types: tuberculins, suspensions of tubercle bacilli, extracts of the same organisms. The complexity of the chemical composition of tuberculin (38) and of the tubercle bacillus (39) is responsible for the failure to produce an antigen of standard composition and reactivity.

Griffith (40) presented a summary of the results of a number of investigators up to 1927. This summary is reproduced in Table VII.



TABLE VIIComplement Fixation Reaction in Tuberculosis



TABLE VII

| <u>Author</u>                          | <u>Results</u> <sup>★</sup>                                                                         |
|----------------------------------------|-----------------------------------------------------------------------------------------------------|
| Alcock, Douglas & Lucey (1925)         | 90.6% in bacillus-positive cases.<br>36.5% in bacillus-negative cases.                              |
| Calmette and Massol (1914)             | 92.0% in proven cases.                                                                              |
| Caulfield (1911)                       | 56.0% in proven cases.                                                                              |
| Cooke (1921)                           | 80.0% in children.                                                                                  |
| Delille, Hillemand & Lestocquay (1922) | 85.0% in bacillus-positive cases.                                                                   |
| Dudgeon, Meck & Weir (1913)            | 84-89% in pulmonary cases.<br>57% in surgical cases.                                                |
| Fidlar (1918)                          | 74-77% in pulmonary cases.                                                                          |
| Fraser (1913)                          | 68% in bacillus-positive cases.                                                                     |
| Fried (1920)                           | 94% in pulmonary cases.                                                                             |
| Hruska and Pfenniger (1921)            | 84.5% in animals (proven post-mortem).<br>2.2% in controls.                                         |
| Ichok, Goldenburg & Fried (1920)       | 66.4% in cases of lupus.<br>16.3% negative in cases of lupus.                                       |
| Kalcher and Sonnenfeld (1924)          | 60.0% in clinically positive cases.                                                                 |
| McIntosh, Fildes & Radcliffe (1914)    | 76.7-90% in pulmonary cases.<br>80.7% in surgical cases.                                            |
| Pai and Rau (1927)                     | 96.7% in clinically positive cases.<br>13.0% in non-tuberculous cases.                              |
| Pinner (1927)                          | 69.0% in acute cases.<br>63.0% in inactive cases.<br>Normal sera, 15%.<br>Wassermann-positive, 29%. |
| Punch (1921 and 1920)                  | 98-100% in pulmonary cases.                                                                         |
| Punch and Gosse (1922)                 | 98% in pulmonary cases.                                                                             |
| Urbain and Fried (1921)                | 35% of syphilitics gave a positive.                                                                 |
| von Wedel (1920)                       | 70% in clinically positive cases.                                                                   |
| Wang and Crockett (1919)               | 84-87% in proven cases.                                                                             |
| Watkins and Boynton (1920)             | 64.6% in proven cases.<br>4.2% in non-tuberculous cases.                                            |

★

This should be interpreted as "Positive Results" unless otherwise indicated.



In 1948 Middlebrook and Dubos (41) described a haemagglutination test for tuberculosis. This has been investigated by a number of workers (42). Although many disconcerting findings have been reported there appears a suggestion of specificity, at least above an arbitrarily selected titre, in the results. A haemolytic modification of the haemagglutination test appeared in 1950 (43).

The haemagglutination technique is the most promising serological test for tuberculosis which has yet appeared, but it will never be generally acceptable until its specificity is much greater and its sensitivity more uniform. Consequently numerous modifications are currently appearing. Of these one of the more interesting is that described by Boyden (44). The antigen used by this worker apparently consists mainly of protein components of tuberculin, whereas the earlier antigens were probably dominantly polysaccharide in nature. In order to adsorb this antigen the red cells used must be first treated with tannic acid.

The antigen in this haemagglutination reaction consists of some component or components of the metabolic products of the tubercle bacillus. There is evidence (45) that at least seven fractions may function as antigenic agents in the agglutination of sensitized sheep erythrocytes.

## II. INTRODUCTION

The haemagglutination reaction of Boyden has not yet been evaluated in a clinical investigation (46). It seemed desirable to have more information on this point before proceeding to any investigation of technical details or any comparison of procedures. Accordingly it was decided to start this work by investigating the applicability of Boyden's technique to clinical material, but it was soon discovered that much



preliminary work had to be carried out on the technique itself.

### III. INVESTIGATION OF THE HAEMAGGLUTINATION TEST

#### BOYDEN'S MODIFICATION

For preliminary work it was decided to test artificially immunized and sensitized rabbit sera. The details of the test as described by Boyden are shown in Appendix IV. Certain comments and initial modifications are shown below.

#### 1. Materials and Methods

Sheep erythrocytes: No differences were detected in blood of different animals. Cells from four animals were used and all were found satisfactory.

Tannic acid: Merck reagent powder (Lot No. 41921) was used throughout this work. Fresh solutions were prepared on the day of testing in buffered saline (pH 7.2).

Antigen: Lederle Old Tuberculin 4x International Standard, lots 2725-33 and 2725-28A, was used throughout this study.

Rabbit anti-B.C.G. and anti-tuberculin serum: Four rabbits (No.'s 326, 365, 380 and 384) were inoculated with 1.0 ml. of a 10-day culture of B.C.G. (Strain No. 637-1, University of Montreal) in Dubos medium. The cultures were centrifuged, the supernate removed and replaced with an equal volume of sterile normal saline.

The inoculations were as follows: 1.0 ml. of the above suspension was injected subcutaneously twice a week for three weeks. Ten days later another injection was given. The animals were bled 5 and 21 days after the last inoculation. Nine weeks later, one further injection was given followed by a bleeding in 5 days.

A second group of four rabbits (No.'s 622, 964, 971 and 974) was



inoculated with Old Tuberculin (Koch and Lederle).

A dose of 0.5 ml. of a 1:50 dilution of O.T. in sterile normal saline was given twice a week for 8 weeks, followed by 10 days' rest. This was followed by a dose of 0.5 ml. of a 1:5 dilution of O.T. twice a week for 3 weeks; two weeks later one more injection of the same dose and dilution was administered, followed by a bleeding 4 days later. The first 6 injections were intravenous, all the rest were subcutaneous.

At the end of the course of inoculations all animals developed a slight skin reaction with local erythema but no induration.

Test tubes: Serological test tubes (7.5 x 100 mm.) were used. As pointed out by Salk (48) it is important that the test tubes have uniformly rounded bottoms in order to obtain the typical "carpet" pattern of the agglutinated erythrocytes in the tannic acid method.

Normal rabbit serum: The serum of apparently healthy normal rabbits was used to prepare normal rabbit serum (N.R.S.) 1:100 and 1:250 dilutions in buffered saline (pH 7.2). The animals were not given the tuberculin skin test because this might have led to the development of specific antibodies.

Sensitization of erythrocytes: Until the optimum dilutions of the tannic acid and of the antigen could be established, tannic acid 1:20,000 in saline, and 0.15 mg. Old Tuberculin (47) per millilitre of 2.5% red cell suspension were used as described by Boyden.

## 2. Initial Observations

Time of centrifugalization: It was found that after treatment with tannic acid, and also after treatment with O.T., the red cells were not adequately thrown down by 3 minutes' centrifugalization at 1500 r.p.m. and 1000 r.p.m. respectively. The time was therefore increased to 6 minutes at the same speeds.

Agglutination pattern: The difference in the appearance of the agglutina-



tion of the sensitized erythrocytes in the haemagglutination tests of Middlebrook and Dubos and of Boyden deserves comment. In the former reaction the agglutinated cells settle to the bottom of the tube in a disc or "button." On tapping the tube gently, the cells in the lower dilutions of the antisera rise in large clumps in the clear medium; in the weaker agglutinating antisera the clumps are smaller and the medium less clear; while in a negative serum the cells rise in a swirling cloud. In the tannic acid method, the haemagglutination pattern is similar to the influenza virus agglutination pattern (48) (Plates II and III), a positive reaction being characterized by a smooth uniform "carpet" on the bottom of the test tube, while a negative reaction shows the cells settled in the shape of a "ring" rather than a solid "button." Around this ring a few cells may form a "halo." Between the strong positive and negative readings, the pattern may vary from an almost complete carpet with a slightly denser aggregation of minute clumps of cells at the periphery (this was read as "+++"); to a ring formation surrounded by finely agglutinated cells (read as "++"); to a pattern slightly larger than the negative ring or almost solid button (read as "+"). The distinction between two plus and one plus readings was difficult to make. For this reason a three plus reading was considered as the end-point of agglutination, any reading between this and a negative result was considered a partial agglutination.

It was also observed that the agglutination pattern of an anti-serum in the tubes with the lower dilutions occasionally was somewhat different from that presented in the tubes with the higher dilutions. Instead of the typical thin "carpet," these tubes showed a denser deposit of the cells with unevenly clumped cells at the edges. The transition from a four or three plus to a negative reading was usually quite abrupt, perhaps through only one or two tubes of dilutions. The virus agglutination type of



pattern was occasionally noted in the higher dilutions of an antiserum in the Middlebrook and Dubos technique. Results of agglutination were read with the aid of a microscope mirror.



PLATES II AND III

Agglutination Patterns

obtained in the

Tannic Acid Technique

of the

Haemagglutination Reaction



PLATE II"Carpet" Pattern(++++ reading)"Ring" Pattern with "Halo"(++ reading)



PLATE III"Button" Formation(Negative)Agglutination Pattern of tannic acid-treated erythrocytes(++ reading)



### 3. Investigation of Individual Factors

The preliminary titrations of the antisera from the rabbits immunized with B.C.G. were carried out using as diluent the sera from two normal rabbits as N.R.S. 1:100 and 1:250. Agglutination was observed in all dilutions of the antisera up to 1:2,560 including the diluent control of N.R.S. 1:100 and sensitized cells. This unsatisfactory result necessitated investigation of factors which might be responsible for non-specific agglutination.

#### (a) Influence of Diluent Serum from Different Rabbits

To determine whether this interference was peculiar to the two rabbit sera first used, the sera from five other normal rabbits were investigated for their protective quality for the tannic acid-tuberculin-treated red blood cells. Table VIII shows the results of the titration of serum #380 before and after immunization using these 5 normal rabbit sera in 1:100 and 1:250 dilutions, as the diluent for the test serum and as the washing and resuspending solution for the sensitized cells, respectively. It is seen that the results are not consistent: N.R.S. #28 caused no agglutination in the diluent control tube; two sera (#32 and #45) agglutinated the sensitized cells strongly, while the remaining two (#76 and #91) produced partial agglutination in the diluent control tube.



TABLE VIIITitration of Rabbit Serum #380before and after Immunization with B.C.G.Using Sera from 5 Normal Rabbits as N.R.S. 1:100, 1:250



TABLE VIII

| 1:100<br>1:250<br>of<br>N.R.S.<br>No. | Serum<br>No. | B e f o r e Immunization |      |      |      |      |       |       |       |         |         |          | A f t e r Immunization |       |       |       |       |         |         |         |          |          |          |           |           |           |          |      |      |      |     |   |
|---------------------------------------|--------------|--------------------------|------|------|------|------|-------|-------|-------|---------|---------|----------|------------------------|-------|-------|-------|-------|---------|---------|---------|----------|----------|----------|-----------|-----------|-----------|----------|------|------|------|-----|---|
|                                       |              | Serum dilutions          |      |      |      |      |       |       |       |         |         |          | Serum dilutions        |       |       |       |       |         |         |         |          |          |          |           |           |           |          |      |      |      |     |   |
|                                       |              | 1:5                      | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | Controls |                        | 1:100 | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 | 1:204,800 | 1:409,600 | Controls |      |      |      |     |   |
|                                       |              | A                        | B    |      |      |      |       |       |       |         |         |          | A                      | B     |       |       |       |         |         |         |          |          |          |           |           |           | A        | B    | C    | D    |     |   |
| 28                                    | 380          | 0                        | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0        | 0                      | +++   | +++   | +++   | ++    | +       | 0       | 0       | 0        | 0        | 0        | 0         | 0         | 0         | 0        | 0    | 0    | 0    | +++ | 0 |
| 32                                    | 380          | +                        | +    | +    | +    | +    | +     | +     | +     | +       | +       | 0        | 0                      | +++   | +++   | ++++  | ++++  | ++++    | ++++    | ++++    | ++++     | ++++     | ++++     | ++++      | ++++      | ++++      | 0        | 0    | ++++ | ++++ |     |   |
| 45                                    | 380          | 0                        | 0    | ++   | ++   | ++   | ++    | ++    | ++    | ++      | ++      | 0        | 0                      | +++   | +++   | +++   | +++   | +++     | +++     | +++     | +++      | +++      | +++      | +++       | +++       | 0         | 0        | +++  | +++  |      |     |   |
| 76                                    | 380          | 0                        | 0    | ++   | ++   | ++   | ++    | ++    | +     | +       | +       | 0        | 0                      | ++    | ++    | +     | +     | +       | +       | +       | +        | +        | +        | +         | +         | 0         | 0        | ++++ | +    |      |     |   |
| 91                                    | 380          | 0                        | 0    | +    | +    | +    | +     | +     | +     | +       | +       | 0        | 0                      | ++    | ++    | ++    | ++    | +       | +       | +       | +        | +        | +        | +         | +         | 0         | 0        | ++++ | +    |      |     |   |

Controls: A = lowest serum dilution + untreated erythrocytes

B = lowest serum dilution + tannic acid-treated erythrocytes

C = saline, pH 7.2 + tannic acid-O.T.-treated erythrocytes

D = diluent (N.R.S. 1:100) + tannic acid-O.T.-treated erythrocytes



Rabbit serum does not appear to be a satisfactory diluent of universal applicability. This led to the search for a diluent for the test sera which would not spontaneously agglutinate the sensitized erythrocytes.

(b) Influence of Electrolyte Concentration on the Stability of Tannic Acid-Old Tuberculin-treated Erythrocytes

It has been shown (49) that erythrocytes after exposure to an antigen are sensitive to the electrolyte concentration in the suspending medium. Therefore, the effect of various concentrations of NaCl and buffer solutions only, and of these saline and buffer solutions in N.R.S. 1:100 on sensitized erythrocytes was determined. The experiment was performed as follows.

Eight NaCl concentrations ranging from 0.5 to 0.85% increasing by 0.05%, buffered at pH 7.2 and pH 6.4 (with 0.15 M  $\text{Na}_2\text{HPO}_4$  and 0.15 M  $\text{KH}_2\text{PO}_4$ ) and buffer solutions of pH 7.2 and pH 6.4 were used. Erythrocytes were washed three times in normal saline, then each of 9 aliquots of a 2.5% cell suspension was put through the process of sensitization using each saline concentration and buffer solution to the stage of washing the tannic acid-O.T.-treated cells. Each aliquot of cells was then divided into two portions, one was washed and resuspended in a buffered saline (pH 7.2), and the second was washed and resuspended in N.R.S. 1:250 prepared with saline of the same concentration and pH. This procedure was repeated with each saline concentration and the buffer (pH 7.2); then 0.05 ml. quantities of untreated, tannic acid-treated, and tannic acid-O.T.-treated cells were mixed with 0.5 ml. quantities of the salt concentrations and buffer solution alone, and with the same solutions as N.R.S. 1:100. The tubes were shaken immediately, allowed to remain at room temperature for 2 hours and then held in the refrigerator overnight.

The results read the following morning are shown in Tables IX (a) and IX (b). It was found that erythrocytes altered by tannic acid alone, and by tannic acid and tuberculin were agglutinated by all saline solutions and by the buffer solution without normal rabbit serum. Erythrocytes once exposed to tannic acid are so altered that the button they form is not as sharply defined as that of untreated cells. All the N.R.S. 1:100 solutions agglutinated the sensitized cells to the same extent (Table IX (b)). It appears that the various concentrations of electrolyte per se do not



influence the sensitized cells to different degrees, at least as far as was shown in this experiment.



TABLE IX (a)Effect ofVarious Salt Concentrations and Buffer (pH 7.2) Solutionon Untreated, Tannic Acid-treated, and Tannic Acid-O.T.-treated ErythrocytesTABLE IX (b)Effect ofN.R.S. 1:100 in Various Salt Concentrations and in Buffer Solution (pH 7.2)on Untreated, Tannic Acid-treated and Tannic Acid-O.T.-treated Erythrocytes



TABLE IX (a)

| 0.05 ml. of 2.5%<br>suspension of:        | 0.5 ml. salt concentrations, pH 7.2,<br>in % |      |      |      |      |      |      |      | 0.5 ml. buffer<br>pH 7.2<br>0.15 M |
|-------------------------------------------|----------------------------------------------|------|------|------|------|------|------|------|------------------------------------|
|                                           | 0.5                                          | 0.55 | 0.6  | 0.65 | 0.7  | 0.75 | 0.8  | 0.85 |                                    |
| untreated erythrocytes                    | 0                                            | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0                                  |
| tannic acid-treated<br>erythrocytes       | ++++                                         | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++                               |
| tannic acid-0.T.-<br>treated erythrocytes | ++++                                         | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++                               |

TABLE IX (b)

| 0.05 ml. of 2.5%<br>suspension of:        | 0.5 ml. N.R.S. 1:100 in the various salt concentrations, pH 7.2,<br>in % |      |     |      |     |      |     |      | N.R.S. 1:100<br>in buffer<br>pH 7.2<br>0.15 M. |
|-------------------------------------------|--------------------------------------------------------------------------|------|-----|------|-----|------|-----|------|------------------------------------------------|
|                                           | 0.5                                                                      | 0.55 | 0.6 | 0.65 | 0.7 | 0.75 | 0.8 | 0.85 |                                                |
| untreated erythrocytes                    | 0                                                                        | 0    | 0   | 0    | 0   | 0    | 0   | 0    | 0                                              |
| tannic acid-treated<br>erythrocytes       | 0                                                                        | 0    | 0   | 0    | 0   | 0    | 0   | 0    | 0                                              |
| tannic acid-0.7.-<br>treated erythrocytes | ++                                                                       | ++   | ++  | ++   | ++  | ++   | ++  | ++   | ++                                             |



(c) Protective Effect of Glycerine and Gelatin on Tannic acid-O.T.  
treated Erythrocytes

Saline alone was found unsuitable in these tests as a suspending agent for the sensitized red cells used. Boyden's recommendation of serum suggested the value of a protective colloid. In the experiments previously discussed even homologous serum (N.R.S.) was found to be inefficient, but other colloidal solutions might be better. Accordingly glycerine and gelatin were investigated for their protective possibilities for sensitized cells.

Solutions of 1% and 0.4% glycerine, and 0.5%, 0.1%, 0.05%, 0.02% and 0.01% gelatin were prepared in 0.85% saline (pH 7.2). Suspensions of 2.5% of untreated and tannic acid-treated cells were prepared in the usual manner, as were the tannic acid-O.T. treated cells to the stage of final washing. An aliquot of sensitized cells was washed and resuspended in each of the above solutions, in buffered saline (pH 7.2), and in N.R.S. 1:250. The tests were carried out in the same manner as in the above section.

The results given in Table IX (c) show that gelatin and glycerine in the concentrations employed did not protect the sensitized erythrocytes. Agglutination of the tannic acid-treated cells and of the tannic acid-O.T.-treated cells occurred in all tubes except the tube of N.R.S. 1:100.



TABLE IX (c)Effect of

Various Concentrations of Gelatin and Glycerine, Saline and N.R.S. 1:100  
on Untreated, Tannic Acid-treated and Tannic Acid-O.T.-treated Erythrocytes







(d) Protective Effect of N.R.S. Absorbed with Sensitized Erythrocytes

Up to this time the N.R.S. used as a diluent had been absorbed with washed packed sheep erythrocytes to remove the heterophile antibodies. The possibility that the non-specific agglutination of the tannic acid-0.T.-treated cells might be caused by certain protein components in the rabbit serum having an affinity for protein antigens on the sheep cell surface was considered. Therefore, normal rabbit serum was absorbed with an equal volume of washed, packed sensitized cells instead of the washed, packed untreated cells. The mixing of the serum with these cells was done very carefully because the sensitized cells were very fragile.

Suspensions (2.5%) of untreated, tannic acid-treated and tannic acid-0.T.-treated cells were prepared in the customary manner, one aliquot of sensitized cells was washed and resuspended in 1:250 N.R.S. absorbed with washed packed cells, and a second aliquot was washed and resuspended in 1:250 of the same N.R.S. absorbed with the sensitized cells. N.R.S. 1:100 was prepared with the two kinds of absorbed serum. Then 0.05 ml. quantities of 2.5% suspensions of untreated, tannic acid-treated and sensitized cells were added to 0.5 ml. of the corresponding N.R.S. 1:100. The tubes were incubated and read in the routine manner.

The results of this experiment are shown in Table IX (d).

TABLE IX (d)

Protective Effect of 1:100 and 1:250 N.R.S. Absorbed with Washed Packed Erythrocytes and with Washed Packed Sensitized Erythrocytes on Erythrocytes Treated with Tannic Acid and Tuberculin

| 2.5% suspension of                    | N.R.S. 1:100<br>absorbed with<br>washed packed<br>cells | N.R.S. 1:100<br>absorbed with<br>washed packed<br>sensitized cells | 0.85% saline<br>(pH 7.2) |
|---------------------------------------|---------------------------------------------------------|--------------------------------------------------------------------|--------------------------|
| untreated erythrocytes                | 0                                                       | 0                                                                  | 0                        |
| tannic acid-treated erythrocytes      | +                                                       | 0                                                                  | +++                      |
| tannic acid-0.T.-treated erythrocytes | +                                                       | 0                                                                  | ++++                     |



It was thus demonstrated that sensitized erythrocytes were not spontaneously agglutinated in 1:100 N.R.S. absorbed with sensitized cells. This serum was stored in the refrigerator until it could be used in actual tests. Two days later titrations of sera from tuberculous patients using this N.R.S. were carried out. In these titrations the diluent provided satisfactory protection for the tannic acid-O.T.-treated cells since no agglutination was observed in the diluent control tube. The results are shown in Table X (a).



TABLE X (a)

Tannic Acid Haemagglutination Titration of Tuberculous Human Sera,  
Using 1:100 and 1:250 N.R.S. Absorbed With Sensitized Erythrocytes



TABLE X (a)

| No.<br>of<br>Serum | Serum dilutions |       |       |       |         |         |         |          |          | Controls |   |    |     |   |
|--------------------|-----------------|-------|-------|-------|---------|---------|---------|----------|----------|----------|---|----|-----|---|
|                    | 1:100           | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | A | B  | C   | D |
| 1                  | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | +++      | ++       | 0 | ++ | +++ | 0 |
| 2                  | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | ++       | 0        | 0 | ++ | ++  |   |
| 3                  | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | ++       | 0        | 0 | ++ | ++  |   |
| 4                  | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | ++       | 0        | 0 | 0  | 0   |   |
| 5                  | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | ++       | 0        | 0 | 0  | 0   |   |
| 6                  | ++              | +++   | +++   | +++   | ++      | ++      | ++      | ++       | ++       | 0        | 0 | ++ | ++  |   |
| 7                  | +               | +++   | +++   | +++   | +       | +       | +       | +        | +        | 0        | 0 | ++ | ++  |   |
| 8                  | 0               | +     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0 | 0  | 0   |   |
| 9                  | 0               | +     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0 | 0  | +   |   |
| 10                 | ++              | +++   | +++   | +++   | ++      | ++      | ++      | ++       | ++       | 0        | 0 | 0  | ++  |   |
| 11                 | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | ++       | ++       | 0 | ++ | ++  |   |
| 12                 | +++             | +++   | +++   | +++   | +++     | +++     | +++     | +++      | ++       | ++       | 0 | ++ | ++  |   |



The maximum reaction was three plus. None of the reacting sera showed the typical four plus "carpet" pattern, three sera (No.'s 6, 9 and 10) produced only partial agglutination and one (#8) was negative. Rabbit anti-serum #326 which was tested at the same time, showed typical four plus agglutination even in the tubes with the lower dilutions and had a titre of 1:64,000. In this experiment the absorbed N.R.S. again appeared to be a satisfactory diluent in that no non-specific agglutination occurred.

Serum from the same normal rabbit but absorbed with a different lot of sensitized cells 5 days earlier and stored in the refrigerator was used as the diluent N.R.S. 1:100 in the preliminary titration of the rabbit sera (No.'s 326, 365, 380 and 384) before and after immunization with B.C.G. The results of these haemagglutination tests were unexpected. The sensitized cells in the diluent control tube were strongly agglutinated, thus invalidating the results of the titrations.

In view of this contradictory evidence of the efficacy of absorbing the diluent normal rabbit serum with sensitized cells, several further haemagglutination tests were carried out, mainly on rabbit sera. Sometimes control tubes remained satisfactory, sometimes spontaneous cell agglutination occurred. The results were quite irregular and quite inexplicable, but altogether they indicated that the solution to the diluent problem had not yet been found.

(e) Protective Effect of N.R.S. Absorbed with B.C.G.

In case the non-specific agglutination observed repeatedly in the diluent control tube might be caused by some protein fraction or fractions in the normal rabbit serum adsorbing onto the antigen-coated cell surface, an attempts was made to absorb normal rabbit serum with killed B.C.G. Five individual rabbit sera were used. This experiment was carried out as follows.



Six tubes of 10-day B.C.G. cultures in 9 ml. Dubos medium were used. The organisms were heat killed at 62°C. for 60 minutes. The tubes of cultures were centrifuged for 10 minutes at 2000 r.p.m. The bacillary mass was washed once in sterile normal saline. The sediments were pooled and suspended in 5 ml. buffered saline (pH 7.2). 1.0 ml. of each normal rabbit serum (No.'s 28, 32, 45, 76 and 91) was mixed with 1.0 ml. of the B.C.G. suspension. The mixtures, after 10 minutes at room temperature, were centrifuged for 10 minutes at 2000 r.p.m. The supernatant sera (1:2) were used to prepare N.R.S. 1:100 and 1:250.

Rabbit antiserum #380 and a normal rabbit serum (#1) were selected for testing with the 5 N.R.S. diluents. Parallel titrations were carried out in each case, using diluents absorbed respectively with washed packed erythrocytes and with B.C.G.

It is seen from Table X (b) that absorption with B.C.G. removed the interfering effect from sera #76 and #91, but not from sera #28, #32 and #45. Serum #28 absorbed with washed packed erythrocytes gave a negative diluent control but showed some agglutination when absorbed with B.C.G.



TABLE X (b)

Protective Effect of 5 Individual Normal Rabbit Sera

Absorbed with Washed Packed Sheep Erythrocytes, and with Killed B.C.G.,  
on Sensitized Erythrocytes



TABLE X (b)

| N.R.S. 1:100<br>with serum<br>No. | Dilutions of Rabbit Antiserum #380 |       |       |       |         |         |         |          |          |          | Controls |   | Dilutions of Negative Rabbit Serum #1 |      |      |      |      |       |       |       |         |         | Controls |   |      |     |
|-----------------------------------|------------------------------------|-------|-------|-------|---------|---------|---------|----------|----------|----------|----------|---|---------------------------------------|------|------|------|------|-------|-------|-------|---------|---------|----------|---|------|-----|
|                                   | 1:100                              | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | A        | B | 1:5                                   | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | A        | B | C    | D   |
| 28 absorbed<br>with w.p.r.b.c.    | +++                                | +++   | +++   | +++   | +++     | +++     | +++     | +++      | +++      | 0        | 0        | 0 | 0                                     | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0        | 0 | ++++ | 0   |
| 28 absorbed<br>with B.C.G.        | +++                                | +++   | +++   | +++   | ++      | ++      | ++      | ++       | +        | +        | 0        | 0 | 0                                     | 0    | +    | ++   | ++   | ++    | +++   | +++   | +++     | ++      | 0        | 0 | ++++ | ++  |
| 32 absorbed<br>with w.p.r.b.c.    | ++++                               | ++++  | ++++  | ++++  | ++++    | ++++    | ++++    | ++++     | ++++     | ++++     | 0        | 0 | 0                                     | +++  | +++  | +++  | +++  | +++   | +++   | +++   | +++     | ++++    | 0        | 0 | ++++ | +++ |
| 32 absorbed<br>with B.C.G.        | +++                                | +++   | +++   | +++   | +++     | +++     | +++     | +++      | +++      | +++      | 0        | 0 | 0                                     | 0    | ++   | ++   | ++   | ++    | ++    | ++    | ++      | ++      | 0        | 0 | ++++ | +++ |
| 45 absorbed<br>with w.p.r.b.c.    | ++                                 | +++   | +++   | +++   | +++     | +++     | +++     | +++      | +++      | ++       | 0        | 0 | 0                                     | 0    | 0    | 0    | ++   | ++    | ++    | ++    | ++      | ++      | 0        | 0 | ++++ | +++ |
| 45 absorbed<br>with B.C.G.        | +++                                | +++   | +++   | +++   | ++++    | ++++    | ++++    | ++++     | ++++     | ++++     | 0        | 0 | 0                                     | 0    | +++  | +++  | +++  | +++   | +++   | +++   | +++     | +++     | 0        | 0 | ++++ | +++ |
| 76 absorbed<br>with w.p.r.b.c.    | ++                                 | +++   | +++   | +++   | +++     | +++     | +++     | +++      | +++      | ++       | 0        | 0 | -                                     | -    | --   | --   | --   | --    | --    | --    | --      | --      | 0        | 0 | +++  | ++  |
| 76 absorbed<br>with B.C.G.        | 0                                  | +++   | +++   | +++   | +++     | +++     | 0       | 0        | 0        | 0        | 0        | 0 | 0                                     | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0        | 0 | ++++ | 0   |
| 91 absorbed<br>with w.p.r.b.c.    | +++                                | +++   | +++   | +++   | ++++    | +++     | +++     | ++       | ++       | ++       | 0        | 0 | -                                     | -    | --   | --   | --   | --    | --    | --    | --      | --      | 0        | 0 | ++++ | +++ |
| 91 absorbed<br>with B.C.G.        | ++                                 | ++    | +++   | +++   | +++     | +++     | +++     | +++      | ++       | 0        | 0        | 0 | 0                                     | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0        | 0 | ++++ | 0   |

w.p.r.b.c. = washed packed sheep red blood cells.



It should be mentioned that the normal rabbit sera were not absorbed with washed packed sheep erythrocytes prior to absorption with B.C.G. Had this been done, the interfering effect might have been removed from sera #32 and #45; on the other hand, the substance causing the non-specific effect in sera #76 and #91 was removed by absorption with B.C.G. alone.

The absorption of N.R.S. with B.C.G. was not continued because of lack of B.C.G. cultures and the inconsistent results obtained.

(f) Effect of Homologous Serum on Sheep Erythrocytes during Storage

In the preceding sections the experiments were designed to find a method of treating normal rabbit serum so that it would stabilize suspensions of sensitized erythrocytes. Before abandoning rabbit serum as the protective agent, one more variable was considered. This was the effect of the homologous serum on sheep cells during storage. It has been well established in virus haemagglutination work (48, 50) that the erythrocytes must be washed free from serum immediately after collection to prevent iso-agglutination.

Therefore, portions of two different lots of sheep cells were washed in normal saline immediately after collection and resuspended in a 10% suspension in Alsever's solution. <sup>(Appendix D)</sup> Aliquots of five normal rabbit sera (No.'s 28, 32, 45, 76 and 91) were absorbed with cells from the first lot and with cells from the same collection stored in the routine manner. A sixth N.R.S. (#1) was similarly treated with cells from the second lot. Sensitized cells prepared from the corresponding lots of cells were then exposed to the respective 1:100 N.R.S.

The results (Table X (c)) were not uniform. Although satisfactory in certain cases, the serum-free method of preserving sheep cells was not significantly superior to the routine procedure.

Thus all methods with normal rabbit serum failed completely or partially.



TABLE X (c)The Agglutinability in N.R.S. of Sensitized Sheep CellsPreserved with and without Homologous Serum



TABLE X (c)

| Sheep erythrocytes preserved by the routine procedure |  |                     |     | Sheep erythrocytes in 10% suspension in Alsever's solution |    |                     |     |
|-------------------------------------------------------|--|---------------------|-----|------------------------------------------------------------|----|---------------------|-----|
| 1st Collection                                        |  | 2nd Collec-<br>tion |     | 1st Collection                                             |    | 2nd Collec-<br>tion |     |
|                                                       |  | 1:100 of N.R.S. No. |     |                                                            |    |                     |     |
| Suspension of                                         |  | 28                  | 32  | 45                                                         | 76 | 91                  | 1   |
| sensitized erythrocytes                               |  | 0                   | +++ | ++                                                         | 0  | 0                   | +++ |
| Suspension of sensitized erythrocytes                 |  |                     |     | sensitized erythrocytes                                    |    |                     |     |



(g) Protective Effect of Bovine Albumin<sup>\*</sup> Solution on Tannic acid-O.T.-  
treated Erythrocytes

The failure of rabbit serum as a stabilizing colloidal diluent led one to wonder if serum from any other animal species could be used. Guinea pig serum was found to be less effective than rabbit serum. Bovine serum was available but might be objected to on account of the possible presence of specific tuberculosis antibodies. Bovine albumin, however, did not seem to be subject to the same objection and was commercially available. A preliminary experiment with concentrations of albumin ranging from 2 to 6% in buffered saline (pH 7.2) indicated that 2 to 3.5% solutions allowed the sensitized cells to settle in a button or small ring pattern surrounded by a faint halo. Stronger solutions produced some agglutination. Table X (d) shows the results of the effect of bovine albumin solutions, N.R.S. 1:100 absorbed with sensitized cells and N.R.S. 1:100 absorbed with tannic acid-treated cells, and buffered saline (pH 7.2) on untreated, tannic acid-treated and sensitized cells.

\* See page 84.



TABLE X (d)

Protective Effect of Bovine Albumin Solutions

on Sensitized Erythrocytes

Compared to the Effects of N.R.S. 1:100

Absorbed with Sensitized and with Tannic Acid-treated Cells



TABLE X (d)

| 2.5% suspensions of:                  | % of bovine albumin in buffered saline (pH 7.2) |     |   |     |   |     |   | 1:100 N.R.S. absorbed with sens. r.b.c. | 1:100 N.R.S. absorbed with tannic acid-treated r.b.c. | Buffered saline pH 7.2 |
|---------------------------------------|-------------------------------------------------|-----|---|-----|---|-----|---|-----------------------------------------|-------------------------------------------------------|------------------------|
|                                       | 2                                               | 2.5 | 3 | 3.5 | 4 | 4.5 | 5 | 6                                       |                                                       |                        |
| untreated erythrocytes                | 0                                               | 0   | 0 | 0   | 0 | 0   | 0 | 0                                       | 0                                                     | 0                      |
| tannic acid-treated erythrocytes      | 0                                               | 0   | 0 | 0   | 0 | 0   | 0 | +                                       | +++                                                   | ++++                   |
| tannic acid-O.T.-treated erythrocytes | 0                                               | 0   | 0 | 0   | + | +   | + | ++                                      | +++                                                   | ++++                   |



By further tests it was found that the lowest concentration of bovine albumin which provided adequate protection for the sensitized erythrocytes was 1.9%. Consequently 1.95% was chosen as a suitable concentration for use in the diluent for the test sera and in the washing and suspending solution for the tannic acid-treated cells after exposure to O.T.

(h) Optimum Amounts of Tannic acid and Old Tuberculin for the Sensitization of Sheep Erythrocytes

Up to this point in the investigation of the tannic acid haem-agglutination reaction, the tannic acid dilution (1:20,000) and the amount of tuberculin (0.15 mg. P.P.D. per ml. of 2.5% erythrocyte suspension) as described by Boyden, had been used. Since the tuberculin in this study was Old Tuberculin (Lederle) which is stated to contain 1000 mg./ml. with reference to Standard Tuberculin (47) it was thought worthwhile to determine the optimum amounts of both tannic acid and Old Tuberculin to sensitize the erythrocytes. Five tannic acid solutions varying from 1:10,000 to 1:100,000 were used to treat the erythrocyte suspensions. One aliquot of each of these was then exposed to each of three different amounts of Old Tuberculin.

Six millilitres of a 2.5% suspension of washed sheep cells in buffered saline (pH 7.2) were mixed with 6 ml. of each of the following dilutions of tannic acid prepared in buffered saline (pH 7.2): 1:10,000, 1:20,000, 1:40,000, 1:80,000 and 1:100,000. The mixtures were incubated in the 37°C. water bath for 10 minutes, they were then centrifuged for 6 minutes at 1,500 r.p.m. and the cells were washed once in buffered saline (pH 7.2). After washing, the cells were resuspended in 6 ml. of buffered saline (pH 7.2). Two millilitres of each erythrocyte suspension were added to 8 ml. of buffered saline (pH 6.4) containing 0.3, 0.2 and 0.1 ml. of a 1:100 dilution of Old Tuberculin in saline of the same pH. The mixtures were allowed to stand at room temperature for 15 minutes, after which they were centrifuged for 6 minutes at 1000 r.p.m., the sedimented cells were washed once in bovine albumin (B.A.) 1.95% in buffered saline (pH 7.2). The washed cells were resuspended in 2 ml. of the same solution. Rabbit antiserum #326 was serially diluted in 2-fold steps in B.A. 1.95% beginning with 1:500 dilution of the test serum. Then 0.05 ml. of each cell suspension was added to 0.5 ml. of each tube of the dilutions. With each cell suspension the appropriate controls were included. All tubes were shaken immediately after the addition of the cells, and placed



in the refrigerator overnight, after 2 hours at room temperature. Readings were made the following morning.

The results are shown in Table XI. In this experiment it was observed that the red cells exposed to 1:80,000 and 1:100,000 tannic acid solutions followed by exposure to all three O.T. amounts were more easily resuspended and remained in suspension better than the cells treated with lower dilutions of tannic acid. As a result of this experiment, 1:80,000 tannic acid was selected as most suitable for treatment of red cell suspensions and these were subsequently exposed to a 1:100 O.T. dilution, 0.15 ml. per ml.



TABLE XI

Optimum Amounts  
of Tannic Acid and Old Tuberculin (Lederle)  
for Sensitization of Sheep Erythrocytes



TABLE XI

| 6 ml. of 2.5% r.b.c. suspension in saline (pH 7.2) for tannic acid treatment: |                                                                                 | Dilutions of antiserum #326 in B.A. 1.95% |         |         |         |         |          |          |          |           |           | Controls |   |      |   |
|-------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-------------------------------------------|---------|---------|---------|---------|----------|----------|----------|-----------|-----------|----------|---|------|---|
| 6 ml. of tannic acid                                                          | 0.5 ml. of 2.5% r.b.c. suspension in saline (pH 7.2) for tannic acid treatment: | 1:500                                     | 1:1,000 | 1:2,000 | 1:4,000 | 1:8,000 | 1:16,000 | 1:32,000 | 1:64,000 | 1:128,000 | 1:256,000 | A        | B | C    | D |
| 1:10,000                                                                      | 0.3                                                                             | ++++                                      | ++++    | ++++    | +++     | +++     | ++       | +        | 0        | 0         | 0         | 0        | 0 | +    | 0 |
|                                                                               | 0.2                                                                             | ++++                                      | ++++    | +++     | +++     | ++      | +        | 0        | 0        | 0         | 0         | 0        | 0 | +    | 0 |
|                                                                               | 0.1                                                                             | +++                                       | +++     | ++      | ++      | +       | +        | 0        | 0        | 0         | 0         | 0        | 0 | +    | 0 |
| 1:20,000                                                                      | 0.3                                                                             | ++++                                      | ++++    | ++++    | ++++    | +++     | ++       | +        | 0        | 0         | 0         | 0        | 0 | +    | 0 |
|                                                                               | 0.2                                                                             | ++++                                      | ++++    | ++++    | ++++    | ++      | +        | 0        | 0        | 0         | 0         | 0        | 0 | +    | 0 |
|                                                                               | 0.1                                                                             | +++                                       | +++     | +++     | +++     | +++     | ++       | +        | 0        | 0         | 0         | 0        | 0 | ++   | 0 |
| 1:40,000                                                                      | 0.3                                                                             | ++++                                      | ++++    | ++++    | ++++    | +++     | +++      | +        | 0        | 0         | 0         | 0        | 0 | ++   | 0 |
|                                                                               | 0.2                                                                             | ++++                                      | ++++    | ++++    | ++++    | ++++    | +++      | ++       | 0        | 0         | 0         | 0        | 0 | ++   | 0 |
|                                                                               | 0.1                                                                             | +++                                       | +++     | +++     | ++      | ++      | ++       | ++       | +        | 0         | 0         | 0        | 0 | ++   | 0 |
| 1:80,000                                                                      | 0.3                                                                             | ++++                                      | ++++    | ++++    | ++++    | +++     | +++      | +++      | ++       | 0         | 0         | 0        | 0 | +++  | 0 |
|                                                                               | 0.2                                                                             | ++++                                      | ++++    | ++++    | ++++    | +++     | ++       | ++       | +        | 0         | 0         | 0        | 0 | ++++ | 0 |
|                                                                               | 0.1                                                                             | +++                                       | +++     | +++     | +++     | +++     | ++       | ++       | 0        | 0         | 0         | 0        | 0 | ++++ | 0 |
| 1:100,000                                                                     | 0.3                                                                             | ++++                                      | ++++    | ++++    | ++++    | ++++    | +++      | ++       | +        | +         | 0         | 0        | 0 | +++  | 0 |
|                                                                               | 0.2                                                                             | ++++                                      | ++++    | ++++    | +++     | +++     | +++      | +++      | ++       | +         | 0         | 0        | 0 | +++  | 0 |
|                                                                               | 0.1                                                                             | ++++                                      | ++++    | +++     | ++      | ++      | 0        | 0        | 0        | 0         | 0         | 0        | 0 | ++++ | 0 |

Controls: A = 0.5 ml., 1:500 serum dilution + 0.05 ml. of 2.5% untreated cell suspension.

B = 0.5 ml., 1:500 serum dilution + 0.05 ml. of 2.5% tannic acid-treated cell suspension.

C = 0.5 ml., saline (pH 7.2) + 0.05 ml. of 2.5% sensitized cell suspension.

D = 0.5 ml., diluent (B.A. 1.95%) + 0.05 ml. of 2.5% sensitized cell suspension.



4. Application of the Above Findings to the  
Examination of Experimental Rabbit Sera

Sera from rabbits inoculated with either B.C.G. or O.T. as previously described were used. In this investigation parallel titrations were carried out with the Boyden technique modified as above and with the Middlebrook-Dubos method as modified by Boyden (Appendix III). This method was used with one change. The erythrocytes were sensitized according to the modification of Middlebrook (43): 0.1 ml. of washed packed cells was mixed with 2.4 ml. of a 1:12 O.T. dilution in buffered saline (pH 7.2). Sera were serially diluted in 2-fold steps in saline of the same pH.

The results of this experiment (Tables XII (a) and XII (b)) show that the modified tannic acid technique was satisfactory in that positive readings, often to high titres, were demonstrable in inoculated animals and the trouble with spontaneous agglutination of the sensitized red cell suspensions seemed to be eliminated.



TABLE XII (a)

Titration of Sera from Rabbits  
before and after O.T. Inoculations



TABLE XII (a)

| Tannic acid technique                               |      |      |      |      |      |       |       |       |         |         |       |                  |    |                                                    |       |       |         |         |         |          |          |          |           |           |   |          |   |     |   |
|-----------------------------------------------------|------|------|------|------|------|-------|-------|-------|---------|---------|-------|------------------|----|----------------------------------------------------|-------|-------|---------|---------|---------|----------|----------|----------|-----------|-----------|---|----------|---|-----|---|
| Before Inoculation<br>Serum dilutions in B.A. 1.95% |      |      |      |      |      |       |       |       |         |         |       | Con-<br>trols    |    | After Inoculation<br>Serum dilutions in B.A. 1.95% |       |       |         |         |         |          |          |          |           |           |   | Controls |   |     |   |
| No.                                                 | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:100 |                  |    | 1:200                                              | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 | 1:204,800 | A |          |   |     |   |
| 622                                                 | 0    | 0    | ++   | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0     | 0                | 0  | +++                                                | +++   | +++   | ++++    | ++++    | +++     | ++       | ++       | 0        | 0         | 0         | 0 | 0        | 0 | +++ | 0 |
| 964                                                 | 0    | +    | +    | +    | +    | +     | 0     | 0     | 0       | 0       | 0     | 0                | 0  | ++++                                               | ++++  | ++++  | ++++    | ++++    | +++     | ++       | +        | 0        | 0         |           |   | 0        | 0 |     |   |
| 971                                                 | +++  | +++  | +++  | +++  | +++  | +++   | +++   | +++   | +++     | +       | 0     | 0                | 0  | ++++                                               | ++++  | ++++  | ++++    | ++++    | +++     | +++      | +++      | +        | 0         |           |   | 0        | 0 |     |   |
| 974                                                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     | 0                | 0  | ++++                                               | ++++  | ++++  | ++++    | ++++    | +++     | +        | 0        | 0        | 0         |           |   | 0        | 0 |     |   |
|                                                     |      |      |      |      |      |       |       |       |         |         |       |                  |    |                                                    |       |       |         |         |         |          |          |          |           |           |   |          |   |     |   |
| Middlebrook-Dubos technique                         |      |      |      |      |      |       |       |       |         |         |       |                  |    |                                                    |       |       |         |         |         |          |          |          |           |           |   |          |   |     |   |
| Before Inoculation<br>Serum Dilutions               |      |      |      |      |      |       |       |       |         |         |       | Control<br>Serum |    | After Inoculation<br>Serum Dilutions               |       |       |         |         |         |          |          |          |           |           |   | Controls |   |     |   |
|                                                     | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5   |                  |    | 1:10                                               | 1:20  | 1:40  | 1:80    | 1:160   | 1:320   | 1:640    | 1:1,280  | 1:2,560  | Saline    | Serum     |   |          |   |     |   |
| 622                                                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     | +                | +  | 0                                                  | 0     | 0     | 0       | 0       | 0       | 0        | 0        |          |           | 0         | 0 |          |   |     |   |
| 964                                                 | ++++ | ++++ | +++  | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     | +++              | ++ | ++                                                 | +     | 0     | 0       | 0       | 0       | 0        | 0        |          |           | 0         |   |          |   |     |   |
| 971                                                 | +    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     | ++               | ++ | +                                                  | 0     | 0     | 0       | 0       | 0       | 0        | 0        |          |           | 0         |   |          |   |     |   |
| 974                                                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     | 0                | 0  | 0                                                  | 0     | 0     | 0       | 0       | 0       | 0        |          |          | 0         |           |   |          |   |     |   |



TABLE XII (b)

Titration of Sera from Rabbits  
before and after B.C.G. Immunization



[illegible][illegible]



The question of specificity of the findings in relation to tuberculous infection still remains open to question. Most obvious, however, is the difference between the readings from the tannic acid technique and the Middlebrook-Dubos method. By the former method sera from rabbits inoculated with O.T. showed considerably higher titres. Sera from B.C.G. inoculated animals, however, showed little difference between the two methods. In both sets the occasional high titre recorded in pre-inoculation specimens was disconcerting since the rabbits were apparently healthy and certainly were not affected with any form of recognized tuberculosis.

Absorption of Pre-inoculation Rabbit Sera with Bovine Erythrocytes

The appearance of agglutination in the sera from rabbits before the injections with B.C.G. and O.T. were started, raised the question of a possible reaction between some elements or fractions in the rabbit serum and the bovine albumin in the solution used as a protective agent for the sensitized erythrocytes.

Since absorption with bovine albumin was scarcely feasible, a tentative alternative was tried. Pre-inoculation sera #380 and #971 which produced the strongest reactions were absorbed first with washed packed sheep cells and then with washed packed bovine erythrocytes. The results of the titration of these sera diluted in B.A. 1.95% are shown in Table XII (c).

The further absorption with bovine cells had practically no effect on the "non-specific?" agglutination. The slight difference in degree of agglutination is obviously of no significance.



TABLE XII (c)

Titration of Pre-inoculation Sera  
Absorbed with Sheep Erythrocytes Only,  
and with Both Sheep and Bovine r.b.c.



TABLE XII (c)

| Serum                                            | Serum dilutions in B.A. 1.95% |      |       |       |       |         |         |         |          |          | Controls |   |     |   |
|--------------------------------------------------|-------------------------------|------|-------|-------|-------|---------|---------|---------|----------|----------|----------|---|-----|---|
|                                                  | 1:40                          | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | 1:5,120 | 1:10,240 | 1:20,480 | A        | B | C   | D |
| 380 (routine absorption with sheep r.b.c. only)  | 0                             | 0    | 0     | 0     | ++    | ++      | ++      | +       | +        | 0        | 0        | 0 | +++ | 0 |
| 380 - absorbed with both sheep and bovine r.b.c. | 0                             | 0    | 0     | +     | +     | ++      | ++      | +       | 0        | 0        | 0        | 0 |     |   |
| 971 (routine absorption with sheep r.b.c. only)  | 0                             | 0    | 0     | 0     | ++    | ++      | ++      | +       | 0        | 0        | 0        | 0 |     |   |
| 971 - absorbed with both sheep and bovine r.b.c. | 0                             | 0    | 0     | 0     | ++    | ++      | +       | +       | 0        | 0        | 0        | 0 |     |   |



## 5. Summary of the Technical Investigation of Boyden's Haemagglutination Reaction

As a consequence of the investigation of the technical aspects of Boyden's tannic acid technique, certain modifications were introduced.

In order to obtain satisfactory sedimentation of treated erythrocytes the time of centrifugalization was increased to 6 minutes at the originally described speed.

The sera from seven apparently normal rabbits failed to provide consistently adequate protection for the tannic acid-O.T.-treated erythrocytes. Trial of several colloidal solutions revealed that bovine albumin in dilute solutions caused no spontaneous agglutination of the sensitized cells. A 1.95% solution of bovine albumin was therefore chosen as the washing and suspending medium for the sensitized erythrocytes and as the diluent for the test sera.

Treatment with tannic acid 1:80,000 and 0.15 ml. of O.T. 1:100 per millilitre of erythrocyte suspension was found to sensitize the cells satisfactorily. Exposure to lower dilutions of tannic acid made resuspension of the treated cells quite difficult.

The importance of the shape of the test tubes used should be emphasized. It was discovered that the least deviation from a perfectly curved bottom in the test tube affected the formation of the typical "carpet" pattern considerably.

With these modifications the tannic acid technique was now considered ready to be applied to titrations of human sera.



#### IV. CLINICAL TRIAL OF THE MODIFIED BOYDEN HAEMAGGLUTINATION TECHNIQUE

##### 1. Introduction

The modified Boyden technique could not be compared with the technique described by the original author because this would not work in the present writer's hands. The new test, however, was compared with the Middlebrook-Dubos method. Sera from tuberculous and non-tuberculous individuals were studied and some observations made on persons with various degrees of allergy to tubercle antigens. Sera from syphilitic patients, from pregnant women and from apparently normal individuals were used as controls.

A total of 129 human sera were tested. These consisted of:

- (1) 68 tuberculous sera.
  - (2) 10 sera from medical students with negative tuberculin tests 5 months earlier, eight of these had been given B.C.G. vaccine.
  - (3) 3 sera from members of the staff, two with negative and one with positive tuberculin skin tests eight months prior to the haemagglutination test.
  - (4) 15 syphilitic sera.
  - (5) 12 pregnancy cases )
  - (6) 11 normal sera )
- negative by the Kahn Presumptive Test.

The tuberculin skin reactions of the individuals in the last three groups were not known.

##### 2. Materials and Methods

★

These were the same as previously described (pages 39 and 40) with



the following additions or alterations.

Human Sera: Dr. H.H. Stephens of the Aberhart Memorial Sanatorium, Edmonton, kindly supplied sera from tuberculous patients and the clinical data. The syphilitic, normal and pregnancy sera were obtained through the kindness of the Provincial Laboratory of Public Health, Edmonton. Members of the staff of the Department of Bacteriology and medical students of the University of Alberta, Edmonton, generously provided additional blood samples.

Tannic acid: Fresh dilutions of tannic acid 1:80,000 in saline (pH 7.2) were prepared on the day of testing.

Bovine Albumin: Three lots of bovine albumin were used in this study: Bovine Albumin Powder, Fraction V, Control No. H 10406 (Armour) and Bovine Plasma, Fraction V, Control No.'s 416840 and 418441 (Difco). B.A. 1.95% solutions were prepared in saline (pH 7.2) as required from a sterile (Seitz filtered) 10% stock solution in the same saline. The stock solution was stored in the refrigerator.

### 3. Results

#### (a) Titration of Sera from Individuals Suffering from Known Tuberculosis.

The titres of the sera from the tuberculous individuals are grouped according to the classification of the disease in the active or the arrested stage as shown in Table XIII. The non-pulmonary conditions are identified beneath the table. The sera obtained from patients suffering from far advanced pulmonary tuberculosis were all positive by both methods with one exception, #6 had a titre of 1:6,400 by the tannic acid method and was negative by the direct sensitization technique. All but one serum showed higher titres by the modified Boyden technique.



TABLE XIII

Haemagglutination Titres of Tuberculous Sera,  
in the Different Classifications of Tuberculosis



TABLE XIII

| Pulmonary tuberculosis |                |                 |                     |                |                 |         |                |                 | Non-pulmonary tuberculosis |                           |                 |
|------------------------|----------------|-----------------|---------------------|----------------|-----------------|---------|----------------|-----------------|----------------------------|---------------------------|-----------------|
| Far advanced           |                |                 | Moderately advanced |                |                 | Minimal |                |                 |                            |                           |                 |
| No.                    | Titres         |                 | No.                 | Titres         |                 | No.     | Titres         |                 | No.                        | Titres                    |                 |
| Active                 | T.a. technique | M.-D. technique | Active              | T.a. technique | M.-D. technique | Active  | T.a. technique | M.-D. technique | Active                     | T.a. technique            | M.-D. technique |
| 1                      | 1:6,400        | 1:40            | 1                   | 1:6,400        | 1:20            | 1       | 1:6,400        | 1:40            | 1                          | 0                         | 1:80            |
| 2                      | 1:6,400        | 1:160           | 2                   | 1:1,600        | 1:160           | 2       | 0              | 1:40            | 2                          | 1:1,600                   | 1:80            |
| 3                      | 1:6,400        | 1:160           | 3                   | 0              | 1:10            | 3       | 0              | 1:20            | 3                          | 0                         | 1:20            |
| 4                      | 1:6,400        | 1:1,280         | 4                   | 1:2,560        | 1:10            | 4       | 0              | 1:20            | 4                          | 1:12,800                  | 0               |
| 5                      | --*            | 1:40            | 5                   | 1:640          | 1:20            | 5       | 0              | 1:10            | 5                          | 1:640                     | 1:20            |
| 6                      | 1:6,400        | 0               | 6                   | 1:1,250        | 1:40            | Arrest. |                |                 | Arrest.                    |                           |                 |
| 7                      | 1:400          | 1:160           | 7                   | 1:640          | 1:160           | 6       | 0              | 1:40            | 6                          | 1:6,400                   | 1:40            |
| 8                      | >1:2,560**     | --              | 8                   | 1:640          | 1:10            | 7       | 1:1,600        | 1:640           | 7                          | 1:1,280                   | 0               |
| 9                      | >1:2,560       | 1:5             | 9                   | 1:800          | 1:20            | 8       | 0              | 0               |                            |                           |                 |
| 10                     | >1:2,560       | 1:160           | 10                  | 1:400          | 1:20            | 9       | 1:800          | 1:40            |                            |                           |                 |
| 11                     | 1:200          | 1:320           | 11                  | 1:200          | 1:10            | 10      | 1:1,600        | 1:20            | No.                        | Disease                   |                 |
| 12                     | 1:320          | 1:40            | Arrest.             |                |                 | 11      | 1:400          | 1:80            | 1                          | Pott's and Hodgkin's      |                 |
| 13                     | 1:640          | 1:320           | 12                  | 1:12,800       | 1:40            | 12      | 0              | 1:10            | 2                          | " " "                     |                 |
| 14                     | 1:10,240       | >1:2,560        | 13                  | 0              | 1:160           | 13      | 1:640          | 1:20            | 3                          | Addison's                 |                 |
| 15                     | 1:800          | 1:10            | 14                  | 1:3,200        | 1:20            | 14      | 0              | 1:40            | 4                          | Pott's and genito-urinary |                 |
| Arrest.                |                |                 | 15                  | 1:25,600       | 1:5             | 15      | 0              | 1:80            | 5                          | Pott's                    |                 |
| 16                     | 1:640          | 1:160           | 16                  | 1:1,600        | 1:10            | 16      | 0              | 1:640           | 6                          | Pott's and Hodgkin's      |                 |
| 17                     | 1:3,200        | 1:640           | 17                  | 1:12,800       | 1:160           | 17      | 1:80           | 1:40            | 7                          | Lymphadenitis             |                 |
| 18                     | 1:6,400        | 1:640           | 18                  | 1:1,600        | 1:20            | 18      | 0              | 1:20            |                            |                           |                 |
| 19                     | 1:25,600       | 1:80            | 19                  | 1:160          | 1:160           |         |                |                 |                            |                           |                 |
| 20                     | 1:6,400        | 1:160           | 20                  | 1:1,600        | 1:40            |         |                |                 |                            |                           |                 |
|                        |                |                 | 21                  | 0              | 1:20            |         |                |                 |                            |                           |                 |
|                        |                |                 | 22                  | 1:160          | 1:40            |         |                |                 |                            |                           |                 |
|                        |                |                 | 23                  | 1:10,240       | 1:160           |         |                |                 |                            |                           |                 |
|                        |                |                 | 24                  | 1:2,560        | 1:20            |         |                |                 |                            |                           |                 |

★ = not done

★★ = insufficient serum to repeat test in higher dilutions

¢ = "+++" agglutination taken as the end-point

@@ = same patient



In the moderately advanced pulmonary tuberculosis group, three sera were negative by the tannic acid technique but all were positive by the second method. The titres of reacting sera, however, were in general much higher by the tannic acid method.

In the minimal tuberculosis group the reaction tendency evident above becomes emphasized, of eighteen sera examined eleven were negative by the modified Boyden technique while all but one were positive by the Middlebrook-Dubos method. Yet distinctly higher titres were still recorded in the former technique when both were positive.

In the non-pulmonary tuberculosis group five out of seven sera were positive by each method, but only three by both methods. In these the "tannic acid" titre was higher.

The titration of the tuberculous sera is shown in Table XIV (a).



TABLE XIV (a)Titration of Tuberculous Sera

TABLE XIV (a)

| Tannic acid technique                                            |                 |       |       |       |         |         |         |          |          |          |           | Direct sensitization technique |      |      |   |                 |      |      |      |      |       |       |       |         |         |                             |     |   |
|------------------------------------------------------------------|-----------------|-------|-------|-------|---------|---------|---------|----------|----------|----------|-----------|--------------------------------|------|------|---|-----------------|------|------|------|------|-------|-------|-------|---------|---------|-----------------------------|-----|---|
| Classification of<br>Tuberculosis<br>No.<br>Far advanced, Active | Serum dilutions |       |       |       |         |         |         |          |          |          |           | Controls                       |      |      |   | Serum dilutions |      |      |      |      |       |       |       |         |         | Controls<br>Saline<br>Serum |     |   |
|                                                                  | 1:100           | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 |                                |      |      |   | 1:5             | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 |                             |     |   |
|                                                                  |                 |       |       |       |         |         |         |          |          |          |           | A                              | B    | C    | D |                 |      |      |      |      |       |       |       |         |         |                             |     |   |
| 1                                                                | ++++            | ++++  | ++++  | ++++  | ++++    | +++     | +++     | ++       | 0        | 0        | 0         | 0                              | ++   | ++++ | 0 | ++++            | ++++ | +++  | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0                           | 0   | 0 |
| 2                                                                | +++             | +++   | +++   | +++   | +++     | +++     | +++     | ++       | 0        | 0        | 0         | 0                              | ++++ |      |   | ++++            | ++++ | ++++ | ++++ | +++  | +     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 3                                                                | +++             | ++++  | ++++  | ++++  | ++++    | ++++    | +++     | ++       | 0        | 0        | 0         | 0                              | 0    |      |   | ++++            | ++++ | +++  | +++  | ++   | +     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 4                                                                | +++             | ++++  | ++++  | ++++  | ++++    | ++++    | +++     | ++       | 0        | 0        | 0         | 0                              | +++  |      |   | ++++            | ++++ | ++++ | ++++ | ++++ | ++++  | +++   | +++   | ++      | 0       |                             | 0   |   |
| 5                                                                | --              | --    | --    | --    | --      | --      | --      | --       | -        | -        | -         | -                              | --   |      |   | ++++            | ++++ | ++++ | +    | 0    | 0     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 6                                                                | ++++            | ++++  | ++++  | ++++  | ++++    | ++++    | +++     | +        | 0        | 0        | 0         | 0                              | 0    |      |   | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 7                                                                | ++++            | +++   | +++   | +     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0                              | 0    |      |   | ++++            | ++++ | ++++ | ++++ | +++  | ++    | +     | 0     | 0       | 0       |                             | 0   |   |
| 8                                                                | 0               | +     | +     | ++++  | +++     | +++     | --      | --       | -        | -        | -         | 0                              | 0    |      |   | --              | --   | --   | --   | --   | --    | --    | --    | --      | --      | -                           | -   |   |
| 9                                                                | ++++            | ++++  | ++++  | ++++  | ++++    | ++++    | --      | --       | -        | -        | -         | 0                              | 0    |      |   | ++              | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 10                                                               | ++++            | ++++  | ++++  | +++   | +++     | +++     | --      | --       | -        | -        | -         | 0                              | 0    |      |   | ++++            | ++++ | ++++ | ++++ | +++  | +     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 11                                                               | ++++            | +++   | ++    | +     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0                              | 0    |      |   | ++++            | ++++ | ++++ | ++++ | ++++ | ++    | +     | 0     | 0       | 0       |                             | 0   |   |
| 12                                                               | ++++            | ++++  | +++   | ++    | +       | 0       | 0       | 0        | 0        | 0        | 0         | 0                              | 0    |      |   | ++++            | ++++ | ++++ | ++   | 0    | 0     | 0     | 0     | 0       | 0       |                             | 0   |   |
| 13                                                               | ++++            | +++   | +++   | +++   | ++      | ++      | ++      | 0        | 0        | 0        | 0         | 0                              | 0    |      |   | ++++            | ++++ | ++++ | ++++ | ++++ | +++   | +     | +     | 0       | 0       |                             | 0   |   |
| 14                                                               | ++++            | ++++  | ++++  | ++++  | ++++    | ++++    | ++++    | +++      | ++       | 0        | 0         | 0                              | 0    |      |   | ++++            | ++++ | ++++ | ++++ | ++++ | ++++  | ++++  | ++++  | +++     | +++     | +++                         | +++ | 0 |
| 15                                                               | +++             | +++   | +++   | +++   | ++      | +       | 0       | 0        | 0        | 0        | 0         | 0                              | 0    |      |   | +++             | ++   | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       |                             | 0   |   |

(table continued on next page)

| Far advanced,<br>arrested      | 1:100 | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 | A | B   | C | D | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | Serum |  |
|--------------------------------|-------|-------|-------|-------|---------|---------|---------|----------|----------|----------|-----------|---|-----|---|---|------|------|------|------|------|-------|-------|-------|---------|---------|-------|--|
| 16                             | +++   | +++   | +++   | +++   | ++      | +       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | +++  | +++  | ++   | +     | 0     | 0     | 0       | 0       | 0     |  |
| 17                             | ++++  | ++++  | ++++  | ++++  | ++++    | +++     | ++      | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | +++  | +++  | +++   | +     | +     | 0       | 0       | 0     |  |
| 18                             | ++    | +++   | ++++  | ++++  | ++++    | ++++    | +++     | 0        | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | ++++ | ++++ | ++++ | ++    | +     | +     | 0       | 0       | 0     |  |
| 19                             | 0     | 0     | +++   | ++++  | ++++    | ++++    | ++++    | ++++     | +++      | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++++ | ++   | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 20                             | ++++  | ++++  | ++++  | ++++  | +++     | +++     | +++     | +        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++++ | ++++ | ++    | 0     | 0     | 0       | 0       | 0     |  |
| Moderately advanced,<br>Active |       |       |       |       |         |         |         |          |          |          |           |   |     |   |   |      |      |      |      |      |       |       |       |         |         |       |  |
| 1                              | ++++  | ++++  | ++++  | ++++  | ++++    | +++     | +++     | +        | 0        | 0        | 0         | 0 | +++ |   |   | ++   | +    | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 2                              | +++   | +++   | +++   | +++   | +++     | ++      | ++      | 0        | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | +++  | ++   | +    | +     | 0     | 0     | 0       | 0       | 0     |  |
| 3                              | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | +++  | +++  | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 4                              | +     | +     | ++++  | ++++  | ++++    | ++++    | ++      | +        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 5                              | +++   | +++   | +++   | ++    | ++      | +       | +       | +        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 6                              | ++++  | ++++  | ++++  | ++++  | +++     | ++      | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | +++  | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 7                              | +++   | ++++  | ++++  | +++   | ++      | +       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | +++  | +++  | ++   | +     | 0     | 0     | 0       | 0       | 0     |  |
| 8                              | ++++  | ++++  | +++   | +++   | ++      | +       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | +++  | +    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 9                              | ++++  | ++++  | +++   | +++   | ++      | +       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 10                             | ++++  | ++++  | +++   | ++    | ++      | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +    | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |
| 11                             | +++   | +++   | +     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++   | +    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0     |  |

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| Moderately advanced,<br>Arrested | 1:100 | 1:200 | 1:400 | 1:800 | 1:1,600                 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 | A | B   | C | D | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | Saline | Serum |
|----------------------------------|-------|-------|-------|-------|-------------------------|---------|---------|----------|----------|----------|-----------|---|-----|---|---|------|------|------|------|------|-------|-------|-------|---------|---------|--------|-------|
| 12                               | +++   | +++   | ++++  | ++++  | ++++                    | ++++    | +++     | +++      | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | +++  | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 13                               | 0     | 0     | 0     | 0     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | +++  | ++   | +     | 0     | 0     | 0       | 0       | 0      |       |
| 14                               | 0     | +++   | +++   | +++   | +++                     | +++     | ++      | +        | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | +++  | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 15                               | 0     | +++   | +++   | +++   | ++++                    | ++++    | +++     | +++      | +++      | 0        | 0         | 0 | 0   |   |   | ++   | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 16                               | 0     | ++    | ++    | +++   | +++                     | ++      | +       | 0        | 0        | 0        | 0         | 0 | +++ |   |   | +++  | +    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 17                               | 0     | +++   | ++++  | ++++  | ++++                    | ++++    | ++++    | +++      | +        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | ++++ | +++  | +    | +     | 0     | 0     | 0       | 0       | 0      |       |
| 18                               | ++    | +++   | +++   | +++   | +++                     | ++      | ++      | 0        | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 19                               | ++    | +++   | +     | 0     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++++ | ++   | +     | 0     | 0     | 0       | 0       | 0      |       |
| 20                               | +++   | +++   | +++   | +++   | +++                     | ++      | ++      | +        | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | ++++ | +++  | +    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 21                               | 0     | 0     | 0     | 0     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++   | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 22                               | +++   | +++   | ++    | +     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | +++  | +++  | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 23                               | ++++  | ++++  | ++++  | +++   | +++                     | +++     | +++     | +++      | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++++ | +++  | ++    | +     | 0     | 0       | 0       | 0      |       |
| 24                               | ++++  | ++++  | ++++  | ++++  | +++                     | +++     | ++      | +        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | ++   | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| Minimal, Active                  |       |       |       |       |                         |         |         |          |          |          |           |   |     |   |   |      |      |      |      |      |       |       |       |         |         |        |       |
| 1                                | 0     | +     | +     | +++   | +++<br>(1:5 dilution++) | ++++    | ++++    | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | ++   | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 2                                | 0     | 0     | 0     | 0     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | ++   | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 3                                | 0     | 0     | 0     | 0     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | ++   | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 4                                | +     | ++    | ++    | ++    | +                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |
| 5                                | 0     | 0     | 0     | 0     | 0                       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      |       |

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| Minimal, Arrested | 1:100 | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 | A | B   | C | D | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | Saline | Serum |
|-------------------|-------|-------|-------|-------|---------|---------|---------|----------|----------|----------|-----------|---|-----|---|---|------|------|------|------|------|-------|-------|-------|---------|---------|--------|-------|
| 6                 | ++    | ++    | ++    | ++    | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 7                 | ++++  | ++++  | ++++  | ++++  | ++++    | ++      | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++++ | +++  | ++    | ++    | +     | 0       | 0       | 0      | 0     |
| 8                 | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 9                 | 0     | 0     | ++++  | ++++  | ++      | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++   | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 10                | 0     | ++++  | ++++  | ++++  | ++++    | ++      | +       | 0        | 0        | 0        | 0         | 0 | ++  |   |   | ++++ | +++  | +    | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 11                | +++   | +++   | +++   | ++    | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | +++ |   |   | ++++ | ++++ | ++++ | ++++ | +++  | +     | 0     | 0     | 0       | 0       | 0      | 0     |
| 12                | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 13                | ++++  | ++++  | ++++  | ++++  | ++      | ++      | 0       | 0        | 0        | 0        | 0         | 0 | ++  |   |   | ++++ | +++  | ++   | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 14                | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++   | +    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 15                | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | +++  | ++   | +    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 16                | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | ++++ | ++++ | ++++ | ++++ | +++   | +++   | +     | 0       | 0       | 0      | 0     |
| 17                | ++++  | ++    | ++    | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | +++  | +++  | +++  | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 18                | 0     | 0     | 0     | 08    | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0   |   |   | ++++ | +++  | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |

(table continued on next page)



| Non-Pulmonary,<br>Active   | 1:100 | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | 1:12,800 | 1:25,600 | 1:51,200 | 1:102,400 | A | B  | C | D | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | Saline | Serum |
|----------------------------|-------|-------|-------|-------|---------|---------|---------|----------|----------|----------|-----------|---|----|---|---|------|------|------|------|------|-------|-------|-------|---------|---------|--------|-------|
| 1                          | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0  |   |   | ++++ | ++++ | ++++ | +++  | ++   | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 2                          | ++++  | ++++  | ++++  | ++++  | ++++    | ++      | 0       | 0        | 0        | 0        | 0         | 0 | 0  |   |   | ++++ | ++++ | ++++ | ++   | +    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 3                          | 0     | 0     | 0     | 0     | 0       | 0       | 0       | 0        | 0        | 0        | 0         | 0 | 0  |   |   | ++++ | +++  | +    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 4                          | +++   | ++++  | ++++  | ++++  | ++++    | ++++    | ++++    | +++      | ++       | 0        | 0         | 0 | 0  |   |   | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 5                          | +     | ++    | ++    | +++   | ++      | +       | 0       | 0        | 0        | 0        | 0         | 0 | 0  |   |   | ++++ | ++++ | ++   | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| Non-Pulmonary,<br>Arrested |       |       |       |       |         |         |         |          |          |          |           |   |    |   |   |      |      |      |      |      |       |       |       |         |         |        |       |
| 6                          | ++    | +++   | +++   | +++   | +++     | +++     | +++     | ++       | ++       | ++       | 0         | 0 | ++ |   |   | ++++ | +++  | ++   | +    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |
| 7                          | ++++  | ++++  | ++++  | +++   | +++     | ++      | 0       | 0        | 0        | 0        | 0         | 0 | 0  |   |   | +    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0       | 0      | 0     |

★ = Not done. Insufficient serum for both tests.

★★ = Insufficient serum to repeat test in higher dilutions.

★★★ = The titres of certain sera, which were tested more accurately in a wider series of dilutions, are shown here to the nearest approximate point on this table.



### Summary

The modified Boyden haemagglutination technique was applied to test 68 sera from proven cases of tuberculosis. The results were compared with those obtained by the direct sensitization method of Middlebrook and Dubos. In general, the tannic acid technique showed higher titres but also a greater number of negative reactions. The latter fact was especially noticeable in the minimal pulmonary tuberculosis group.

(b) Titration of Sera from Individuals Not Suffering from Known Tuberculosis.

There were fewer positive reactions in this group (Table XIV (b)). This was most obvious with the modified Boyden technique. In this connection a possibly significant difference appeared between the two techniques. The incidence of positive haemagglutination was almost wholly confined to the syphilitic group (all Kahn and Wassermann positive) by the tannic acid technique, whereas there was no such discrimination with the direct sensitization technique. Incidentally, there was no correlation between the degree of reactions in Kahn or Wassermann tests and positive findings in either series.



TABLE XIV (b)

Titration of Sera from Individuals  
not Suffering from Known Tuberculosis



TABLE XIV (b)

| Source of Serum   | No. | Tannic acid technique |      |      |      |      |       |       |       |         |         | Direct sensitization technique |     |   |     |           |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|-------------------|-----|-----------------------|------|------|------|------|-------|-------|-------|---------|---------|--------------------------------|-----|---|-----|-----------|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|                   |     | Serum dilutions       |      |      |      |      |       |       |       |         |         | Serum dilutions                |     |   |     |           |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|                   |     | 1:5                   | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560 | A                              | B   | C | D   | Con-trols |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Syphilis subjects | 1   | 0                     | 0    | +    | +    | +++  | +++   | +++   | +++   | +       | 0       | 0                              | +++ | 0 | +++ | 0         | +++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |



In this group the infection experience of individuals in relation to tuberculosis was unknown and therefore the specificity of the findings in relation to such infection could not be assessed. Certainly the modified Boyden technique seemed to have more relationship to current clinical findings, but it was felt that more information about its relationship to infection and immunity might be obtained from the next group.

(c) Titration of Sera from Individuals with No Disease but with Known Exposure to Tubercle Antigens.

The study of the 13 sera from individuals who had been tuberculin skin tested and 8 of whom had been given B.C.G. vaccine (Table XIV (c)) was undertaken to determine whether some relationship between the development of tuberculin hypersensitivity and the production of circulating antibody might be demonstrated. Of the sera of the 5 persons (No.'s 1, 2, 3, 4, and 5) who were tuberculin skin tested only, 5-7 months earlier, four contained no circulating antibodies detectable by the tannic acid technique, while one (#5) produced a partial agglutination by this method; just the opposite results were obtained by the direct sensitization method, serum #5 was negative and the other four were positive.



TABLE XIV (c)

Titration of Sera from Individuals  
with Known Tuberculin Reactions and B.C.G. Vaccination



Tannic acid technique

TABLE XIV (c) Middlebrook - Dubos technique

| B.C.G. Vaccine<br>5 mos.<br>earlier | O.T. Skin<br>Test | No.              | Serum dilutions |      |      |      |      |       |       |       |         | Controls           |   |   |     | Serum dilutions |      |      |      |      |      |       |       |       | Controls |       |
|-------------------------------------|-------------------|------------------|-----------------|------|------|------|------|-------|-------|-------|---------|--------------------|---|---|-----|-----------------|------|------|------|------|------|-------|-------|-------|----------|-------|
|                                     |                   |                  | 1:5             | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 | 1:2,560            | A | B | C   | D               | 1:5  | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | Saline   | Serum |
| No                                  | Neg.              | 1 <sup>★</sup>   | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 | +++ | 0               | ++++ | ++++ | ++++ | ++++ | ++++ | +++   | +     | 0     | 0        | 0     |
| No                                  | Pos.              | 2 <sup>★</sup>   | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | ++++ | ++++ | +++  | +    | +    | 0     | 0     | 0     | 0        | 0     |
| No                                  | Neg.              | 3 <sup>★</sup>   | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | ++   | +    | +    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| No                                  | Neg.              | 4a <sup>★★</sup> | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | +++  | +++  | +++  | +    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 4b <sup>★★</sup> | 0               | 0    | 0    | 0    | +    | +     | ++    | +     | 0       | 0                  | 0 | 0 |     |                 | +++  | +++  | ++   | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| No                                  | Neg.              | 5a               | 0               | 0    | 0    | 0    | 0    | ++    | ++    | +     | +       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 5b               | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 6a               | 0               | 0    | 0    | 0    | 0    | +++   | +++   | +++   | +++     | ++                 | 0 | 0 |     |                 | +    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 6b               | 0               | 0    | 0    | +    | +    | ++++  | ++++  | +++   | ++      | ++                 | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 7a               | 0               | 0    | 0    | ++   | ++++ | ++++  | ++++  | ++++  | ++++    | ++++ <sup>φφ</sup> | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 7b               | 0               | 0    | 0    | +    | ++++ | ++++  | ++++  | ++++  | ++++    | ++                 | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Neg.              | 8a <sup>φ</sup>  | 0               | 0    | 0    | 0    | 0    | +     | ++    | ++    | +       | 0                  | 0 | 0 |     |                 | ++++ | ++++ | +++  | +    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 8b               | 0               | 0    | +    | ++++ | ++++ | +++   | ++    | ++    | 0       | 0                  | 0 | 0 |     |                 | ++++ | ++++ | ++++ | +++  | ++   | +     | +     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 9a               | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 9b               | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 10a              | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 10b              | 0               | 0    | 0    | 0    | 0    | +     | +     | +     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 11a              | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 11b              | 0               | 0    | 0    | +    | ++   | +++   | +++   | ++    | ++      | 0                  | 0 | 0 |     |                 | +    | +    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 12a              | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | ++++ | ++   | +    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 12b              | 0               | 0    | 0    | +    | +    | +     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | 0    | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
| Yes                                 | Pos.              | 13a              | 0               | 0    | 0    | 0    | 0    | 0     | 0     | 0     | 0       | 0                  | 0 | 0 |     |                 | ++++ | ++   | +    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |
|                                     |                   | 13b              | 0               | 0    | 0    | 0    | +    | ++    | ++    | ++    | +       | 0                  | 0 | 0 |     |                 | ++   | +    | +    | 0    | 0    | 0     | 0     | 0     | 0        | 0     |

★ = 1-3 O.T. skin test 7 months earlier

★★ (a) = blood specimen collected 5 months after B.C.G. vaccination and before O.T. skin test

★★★ (b) = blood specimen collected 11 days after O.T. skin test, with result as indicated

φ = subject #8 was given B.C.G. immediately after negative O.T. skin test, 10 days prior to second haemagglutination test

φφ = 7a insufficient serum to repeat test in higher dilutions



Seven of the 8 B.C.G. subjects developed a positive reaction to tuberculin, but in only two (#6 and #7) of these were antibodies detectable by the tannic acid method, both before and after the O.T. skin test, both, however, were negative by the direct sensitization method. Case #8 was tuberculin negative and his serum taken before skin test gave only a slight agglutination with the modified Boyden method, but was positive by the Middlebrook-Dubos technique. A little over a week after skin test and subsequent B.C.G. vaccination his serum was positive by both methods. Of the remaining 5 sera (No.'s 9, 10, 11, 12 and 13) two (#9 and #10) were negative by both methods before and after the skin test. By the modified Boyden technique the remaining three individuals behaved as might have been expected in that their sera, originally negative, became positive (though feebly in two cases) 11 days after skin test. The findings in these three persons by the Middlebrook-Dubos technique were completely irregular; one was negative before and after skin test, one was positive before the test and negative after, and one was positive on both occasions.

On the basis of these few tests it is impossible to come to any final conclusion, for there does not seem to be any absolute correlation between either variant of the haemagglutination test and tuberculin allergy and our conception of the probable development of humoral immunity to tuberculosis. The modified Boyden technique gave results which could be interpreted as slightly more specific than the Middlebrook-Dubos method, but inexplicable irregularities make further comment impossible.

Further serological studies of the B.C.G. subjects were impossible.

The results of the haemagglutination reaction of the human sera by the two methods are summarized in Table XV.



TABLE XV

Summary of the Results  
of the Haemagglutination Test of Human Sera  
by the Modified Boyden and the Middlebrook-Dubos Methods



TABLE XV

| Source of Sera                             | Total No. of Sera | Tannic acid method |      |          |      | Total No. of Sera | Direct sensitization method |       |          |      |
|--------------------------------------------|-------------------|--------------------|------|----------|------|-------------------|-----------------------------|-------|----------|------|
|                                            |                   | Positive           |      | Negative |      |                   | Positive                    |       | Negative |      |
|                                            |                   | No.                | %    | No.      | %    |                   | No.                         | %     | No.      | %    |
| tuberculous patients                       | 67                | 52                 | 77.6 | 15       | 22.4 | 67                | 63                          | 94.03 | 4        | 5.97 |
| syphilitic subjects                        | 15                | 5                  | 33.3 | 10       | 66.7 | 13                | 11                          | 84.6  | 2        | 15.4 |
| pregnancy cases                            | 12                | 2                  | 16.7 | 10       | 83.3 | 12                | 7                           | 58.3  | 5        | 41.7 |
| normal, including the O.T. tested subjects | 24                | 3                  | 12.5 | 21       | 87.5 | 24                | 12                          | 50.0  | 12       | 50.0 |



(d) Discussion of Results.

The results shown in Table XV seem to rule out the application to clinical diagnosis of any of the above haemagglutination techniques. Certainly there is no absolute correlation with degree, duration or activity of disease; cases of advanced tuberculosis of two months' and seven years' duration respectively show the same titre, a moderately advanced case of one month's duration gives a titre of at least 1:400 while a clinically similar case of 5 years' duration is negative, cases arrested for many years show occasional titres as high as persons with advanced active disease. Yet the tests should not be abandoned without very much more investigation. This applies particularly to the Boyden technique and modifications as described here. This test seems to have a degree of specificity considerably greater than the Middlebrook-Dubos test and suggests that in individual patients a positive finding is more likely to be influenced by variations in humoral response to the activities of tuberculous disease than by adventitious factors which may influence tuberculous as well as normal individuals. Thus it may be that the test described above or further modifications of it may have a possible value in serial application to individual patients and that the waxing and waning of measurable antibodies may have a clinical correlation as yet unknown.

It may seem odd that the Boyden modification, with a specificity demonstrably greater than the Middlebrook-Dubos method, should at the same time apparently have a greater sensitivity as measured by recorded titres. The sensitivities, however, cannot be compared in this way because of the chemical differences in the antigens concerned and the possible difference in the antibodies measured.



## V. Summary of Part II

An attempt was made to apply to the serological investigation of tuberculosis Boyden's newly described haemagglutination test. This differs from the test identified with Middlebrook and Dubos in that the red cells used to adsorb the tuberculin antigen are previously treated with tannic acid. Untreated erythrocytes adsorb polysaccharide components only but tannic acid-treated cells have an additional affinity for protein. Thus the new antigen may have a different specificity.

Early difficulties led to a more intensive study of certain technical features of the test, particularly the antigen diluent. The difficulty of maintaining uniform suspensions of sensitized erythrocytes in diluted normal rabbit serum as described by the original author led to various investigations. It was found that serum from apparently normal rabbits, even after absorption to remove heterophile antibodies, had a variable and unpredictable influence on the stability of sensitized tannic acid-treated sheep cells, agglutination occurring in some but not in others. Attempts to dilute normal rabbit serum in salt solutions of lower electrolyte concentrations than the physiological level (0.85%) or to replace it with substances like glycerine and gelatin were unsuccessful. Preliminary absorption of rabbit serum with sensitized rather than normal erythrocytes gave encouraging results, but further tests showed that this was still unreliable. Other experiments were carried out but the final conclusion was that rabbit serum in any form was unreliable as an antigen diluent. Some form of "protective" diluent was, however, essential to prevent auto-agglutination. Bovine albumin available in uniform commercial form was found to be satisfactory. A suitable resuspending agent having been found it was possible to determine the optimum amounts of tannic acid



and Old Tuberculin necessary in the sensitization process. The selected modifications were introduced into the Boyden technique (Appendix IV).

The modified Boyden haemagglutination test was then applied to the investigation of 129 sera from tuberculous and other individuals and the results were compared with those from parallel tests by the more widely known Middlebrook-Dubos technique.

Complete correlation between the observed titres and the degree, duration and activity of disease could not be established, but the results suggest that the Boyden test is somewhat more specific than the Middlebrook-Dubos test.



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APPENDIX



I.EVALUATION STUDIES IN CANADATABLE XVI (a)Standard Kahn TestSixth Evaluation Study (1952-53)



TABLE XVI (a)

| Laboratory                      | Individuals with diagnoses of syphilis,<br>untreated or treated (approx. 25% seronegative) |          |      |          |      |          |      |             |
|---------------------------------|--------------------------------------------------------------------------------------------|----------|------|----------|------|----------|------|-------------|
|                                 | Total<br>Sera                                                                              | Positive |      | Doubtful |      | Negative |      | Sensitivity |
|                                 |                                                                                            | No.      | %    | No.      | %    | No.      | %    |             |
| L. of H.<br><sup>1st</sup><br>C | 87                                                                                         | 41       | 47.1 | 21       | 24.1 | 25       | 28.8 | 59.2        |
|                                 | 87                                                                                         | 37       | 42.5 | 27       | 31.1 | 23       | 26.4 | 58.1        |
|                                 | 87                                                                                         | 43       | 49.5 | 15       | 17.2 | 29       | 33.3 | 58.1        |
|                                 | 87                                                                                         | 34       | 39.1 | 13       | 14.9 | 40       | 46.0 | 46.6        |
| H                               | 87                                                                                         | 25       | 28.8 | 23       | 26.4 | 39       | 44.8 | 42.0        |
| I                               | 87                                                                                         | 46       | 52.9 | 21       | 24.1 | 20       | 23.0 | 65.0        |
| L. of H.<br>B                   | 86                                                                                         | 41       | 47.7 | 21       | 24.4 | 24       | 27.8 | 59.9        |
|                                 | 86                                                                                         | 40       | 46.5 | 21       | 24.4 | 25       | 29.1 | 58.7        |
|                                 | 86                                                                                         | 33       | 38.4 | 19       | 22.1 | 34       | 39.1 | 49.5        |
|                                 | 86                                                                                         | 49       | 57.0 | 15       | 17.4 | 22       | 25.6 | 65.7        |
| L. of H.<br>A                   | 85                                                                                         | 39       | 45.9 | 21       | 24.7 | 25       | 29.4 | 58.3        |
|                                 | 85                                                                                         | 31       | 36.5 | 20       | 23.5 | 34       | 40.0 | 48.3        |
| L. of H.<br>F                   | 85                                                                                         | 41       | 48.2 | 20       | 23.5 | 24       | 28.3 | 60.0        |
|                                 | 85                                                                                         | 20       | 23.5 | 21       | 24.7 | 44       | 51.8 | 35.9        |

| Laboratory | Individuals with no clinical evidence of syphilis |          |   |          |      |          |       |             |
|------------|---------------------------------------------------|----------|---|----------|------|----------|-------|-------------|
|            | Total<br>Sera                                     | Positive |   | Doubtful |      | Negative |       | Specificity |
|            |                                                   | No.      | % | No.      | %    | No.      | %     |             |
| L. of H.   | 101                                               |          |   |          |      | 101      | 100   | 100.0       |
| A          | 101                                               |          |   |          |      | 101      | 100   | 100.0       |
| C          | 101                                               |          |   | 1        | 0.99 | 100      | 99.01 | 99.5        |
| D          | 101                                               |          |   |          |      | 101      | 100   | 100         |
| E          | 101                                               |          |   | 1        | 0.99 | 100      | 99.01 | 99.5        |
| G          | 101                                               |          |   |          |      | 101      | 100   | 100         |
| I          | 101                                               |          |   | 2        | 1.98 | 99       | 98.02 | 99.0        |
| J          | 101                                               |          |   | 1        | 0.99 | 100      | 99.01 | 99.5        |
| F          | 100                                               |          |   |          |      | 100      | 100   | 100         |
| H          | 100                                               |          |   |          |      | 100      | 100   | 100         |
| B          | 98                                                |          |   |          |      | 98       | 100   | 100         |

★ L. of H. = Laboratory of Hygiene

★★ A - J = code designations for different laboratories



TABLE XVI (b)

Complement Fixation Test

Sixth Evaluation Study (1952-53)



TABLE XVI (b)

| Laboratory | Individuals with diagnoses of syphilis, treated or untreated<br>(approx. 25% sero-negative) |          |      |          |      |             |
|------------|---------------------------------------------------------------------------------------------|----------|------|----------|------|-------------|
|            | Total<br>Sera                                                                               | Positive |      | Doubtful |      | Sensitivity |
|            |                                                                                             | No.      | %    | No.      | %    |             |
| L. of H.   | 86                                                                                          | 56       | 65.1 | 14       | 16.3 | 73.3        |
| D          | 86                                                                                          | 44       | 51.2 | 19       | 22.1 | 62.3        |
| I          | 86                                                                                          | 55       | 64.0 | 13       | 15.1 | 71.6        |
| L. of H.   | 85                                                                                          | 56       | 65.9 | 14       | 16.5 | 74.2        |
| B          | 85                                                                                          | 56       | 65.9 | 10       | 11.8 | 71.8        |
| L. of H.   | 85                                                                                          | 56       | 65.9 | 13       | 15.3 | 73.6        |
| C          | 85                                                                                          | 56       | 65.9 | 13       | 15.3 | 73.6        |
| L. of H.   | 84                                                                                          | 55       | 65.5 | 14       | 16.7 | 73.9        |
| E          | 84                                                                                          | 56       | 66.7 | 11       | 13.1 | 73.3        |
| L. of H.   | 83                                                                                          | 53       | 63.8 | 14       | 16.9 | 72.3        |
| A          | 83                                                                                          | 54       | 65.0 | 8        | 9.7  | 69.9        |
| L. of H.   | 83                                                                                          | 55       | 66.2 | 14       | 16.9 | 74.7        |
| F          | 83                                                                                          | 26       | 31.3 | 10       | 12.1 | 37.4        |
| L. of H.   | 83                                                                                          | 55       | 66.2 | 13       | 15.7 | 74.1        |
| G          | 83                                                                                          | 50       | 60.2 | 9        | 10.8 | 65.6        |
| L. of H.   | 80                                                                                          | 51       | 63.8 | 14       | 17.5 | 72.6        |
| H          | 80                                                                                          | 61       | 76.2 | 9        | 11.3 | 81.9        |
| L. of H.   | 80                                                                                          | 51       | 63.8 | 13       | 16.2 | 71.9        |
| J          | 80                                                                                          | 56       | 70.0 | 9        | 11.3 | 75.7        |
| Laboratory | Individuals with no clinical evidence of syphilis                                           |          |      |          |      |             |
|            | Total<br>Sera                                                                               | Positive |      | Doubtful |      | Specificity |
|            |                                                                                             | No.      | %    | No.      | %    |             |
| L. of H.   | 101                                                                                         |          |      | 1        | 0.99 | 99.5        |
| A          | 101                                                                                         |          |      | 1        | 0.99 | 99.5        |
| B          | 98                                                                                          |          |      |          |      | 100.0       |
| C          | 101                                                                                         |          |      | 1        | 0.99 | 99.5        |
| D          | 101                                                                                         |          |      | 2        | 1.98 | 99.0        |
| E          | 101                                                                                         |          |      | 1        | 0.99 | 99.5        |
| F          | 99                                                                                          |          |      | 1        | 1.02 | 99.5        |
| G          | 101                                                                                         |          |      |          |      | 100.0       |
| H          | 94                                                                                          |          |      | 2        | 2.13 | 98.9        |
| I          | 101                                                                                         |          |      | 1        | 0.99 | 99.5        |
| J          | 100                                                                                         |          |      | 1        | 1.0  | 99.5        |



TABLE XVI (c)

Mazzini Microscopic Flocculation Test

Sixth Evaluation Study (1952-53)



TABLE XVI (c)

| Laboratory | Individuals with diagnoses of syphilis, treated or untreated<br>(approx. 25% sero-negative) |          |      |          |      |          |        |             |       |
|------------|---------------------------------------------------------------------------------------------|----------|------|----------|------|----------|--------|-------------|-------|
|            | Total<br>Sera                                                                               | Positive |      | Doubtful |      | Negative |        | Sensitivity |       |
|            |                                                                                             | No.      | %    | No.      | %    | No.      | %      |             |       |
| L. of H.   | 87                                                                                          | 72       | 82.8 | 7        | 8.0  | 8        | 9.2    | 86.8        |       |
| C          | 87                                                                                          | 59       | 67.8 | 19       | 21.8 | 9        | 10.4   |             | 78.7  |
| E          | 87                                                                                          | 59       | 67.8 | 16       | 18.4 | 12       | 13.8   |             | 77.0  |
| L. of H.   | 86                                                                                          | 71       | 82.6 | 7        | 8.1  | 8        | 9.3    | 86.6        |       |
| B          | 86                                                                                          | 67       | 77.9 | 10       | 11.6 | 9        | 10.5   |             | 83.7  |
| L. of H.   | 82                                                                                          | 67       | 81.7 | 7        | 8.5  | 8        | 9.8    | 85.9        |       |
| J          | 82                                                                                          | 65       | 79.3 | 8        | 9.8  | 9        | 10.9   |             | 84.2  |
| L. of H.   | 81                                                                                          | 69       | 85.2 | 6        | 7.4  | 6        | 7.4    | 88.9        |       |
| D          | 81                                                                                          | 37       | 45.7 | 26       | 32.1 | 18       | 22.2   |             | 61.7  |
| Laboratory | Individuals with no clinical evidence of syphilis                                           |          |      |          |      |          |        |             |       |
|            | Total<br>Sera                                                                               | Positive |      | Doubtful |      | Negative |        | Specificity |       |
|            |                                                                                             | No.      | %    | No.      | %    | No.      | %      |             |       |
| L. of H.   | 101                                                                                         |          |      |          |      | 101      | 100.00 | 100.0       |       |
| B          | 98                                                                                          |          |      | 2        | 2.04 | 96       | 97.96  |             | 99.0  |
| C          | 101                                                                                         |          |      |          |      | 101      | 100.00 |             | 100.0 |
| D          | 95                                                                                          |          |      |          |      | 95       | 100.00 |             | 100.0 |
| E          | 101                                                                                         |          |      |          |      | 101      | 100.00 |             | 100.0 |
| J          | 100                                                                                         |          |      | 5        | 5.00 | 95       | 95.00  |             | 97.5  |



TABLE XVI (d)V.D.R.L. Slide TestSixth Evaluation Study (1952-53)



TABLE XVI (d)

| Laboratory | Individuals with diagnoses of syphilis, treated or untreated<br>(approx. 25% sero-negative) |          |      |          |      |          |      |             |
|------------|---------------------------------------------------------------------------------------------|----------|------|----------|------|----------|------|-------------|
|            | Total                                                                                       | Positive |      | Doubtful |      | Negative |      | Sensitivity |
|            |                                                                                             | No.      | %    | No.      | %    | No.      | %    |             |
| L. of H.   | 87                                                                                          | 69       | 79.3 | 5        | 5.7  | 13       | 15.0 | 82.2        |
| E          | 87                                                                                          | 67       | 77.0 | 10       | 11.5 | 10       | 11.5 | 82.8        |
| L. of H.   | 86                                                                                          | 68       | 79.1 | 5        | 5.8  | 13       | 15.1 | 82.0        |
| D          | 86                                                                                          | 59       | 68.6 | 15       | 17.4 | 12       | 14.0 | 77.3        |
| L. of H.   | 85                                                                                          | 60       | 80.0 | 5        | 5.9  | 12       | 14.1 | 83.0        |
| B          | 85                                                                                          | 66       | 77.6 | 9        | 10.6 | 10       | 11.8 | 82.9        |
| L. of H.   | 84                                                                                          | 66       | 78.6 | 5        | 5.9  | 13       | 15.5 | 81.6        |
| A          | 84                                                                                          | 52       | 61.9 | 14       | 16.7 | 18       | 21.4 | 70.3        |
| L. of H.   | 82                                                                                          | 64       | 78.0 | 5        | 6.1  | 13       | 15.9 | 81.1        |
| F          | 82                                                                                          | 55       | 67.1 | 12       | 14.6 | 15       | 18.5 | 74.4        |
| L. of H.   | 80                                                                                          | 62       | 77.5 | 5        | 6.2  | 13       | 16.3 | 80.6        |
| J          | 80                                                                                          | 67       | 83.7 | 6        | 7.5  | 7        | 8.8  | 87.5        |
| L. of H.   | 11                                                                                          | 7        | 63.6 | 1        | 9.1  | 3        | 27.3 | 68.2        |
| C          | 11                                                                                          | 6        | 54.5 | 3        | 27.3 | 2        | 18.2 | 68.2        |

| Laboratory | Individuals with no clinical evidence of syphilis |          |      |          |      |          |        |             |
|------------|---------------------------------------------------|----------|------|----------|------|----------|--------|-------------|
|            | Total                                             | Positive |      | Doubtful |      | Negative |        | Specificity |
|            |                                                   | No.      | %    | No.      | %    | No.      | %      |             |
| L. of H.   | 101                                               |          |      |          |      | 101      | 100.00 | 100.0       |
| A          | 97                                                |          |      |          |      | 97       | 100.00 | 100.0       |
| B          | 98                                                |          |      |          |      | 98       | 100.00 | 100.0       |
| C          | 18                                                |          |      |          |      | 18       | 100.00 | 100.0       |
| D          | 101                                               |          |      |          |      | 101      | 100.00 | 100.0       |
| E          | 101                                               |          |      |          |      | 101      | 100.00 | 100.0       |
| F          | 94                                                |          |      | 1        | 1.06 | 93       | 98.94  | 99.50       |
| J          | 98                                                | 1        | 1.02 | 2        | 2.04 | 95       | 96.94  | 98.00       |



II.ALSEVER'S SOLUTION (51)

Dextrose ..... 2.05 gm.

Sodium citrate ..... 0.8 gm.

Sodium chloride ..... 0.42 gm.

Distilled water ..... 100.00 ml.

Adjust to pH 6.1 with 10% citric acid solution. Sterilize in the autoclave at 10 pounds for 15 minutes.



### III.

#### BOYDEN'S MODIFICATION OF THE HAEMAGGLUTINATION TEST OF MIDDLEBROOK AND DUBOS (44)

One volume of a 2.5% suspension of sheep erythrocytes in saline is added to 5 volumes of saline containing 0.1 volume of P.P.D. Weybridge, and incubated for 2 hours at 37°C.

Centrifuge the mixture, wash the sedimented cells once in saline. Resuspend the cells to the original volume making a 2.5% suspension. Prepare 0.5 ml. of a 2.5% suspension of untreated erythrocytes for the control of untreated cells with the lowest dilution of the test serum.

Test sera are inactivated for 30 minutes at 56°C., 1 volume of serum is absorbed with 1 volume of washed packed sheep erythrocytes for 10 minutes at room temperature. The sheep cells are removed by centrifuging the sera for 10 minutes at 2000 r.p.m.

Dilutions of the test serum are made in saline in 3-fold steps in 0.5 ml. volumes.

Add 0.05 ml. of the 2.5% suspension of the sensitized sheep cells to each tube.

Controls: (1) Saline 0.5 ml. + 0.05 ml. sensitized cells.

(2) Serum 1:5 dilution, 0.5 ml. + 0.05 ml. untreated cells.

Shake tubes 30 minutes after addition of the cells. Leave tubes at room temperature for 5 hours, then place them in the refrigerator at 2-4°C. overnight. In the morning incubate the tubes for 5 minutes at 37°C.

Readings are made by gently shaking the tubes to observe clumping.



IV.

THE BOYDEN TANNIC ACID METHOD  
OF THE HAEMAGGLUTINATION TEST (44)

Sheep erythrocytes. Sheep blood is collected aseptically in 1.2 volumes of modified Alsever's solution (Appendix II). The blood is stored for 3 days before using and is not used after 3 weeks. It is stored in the refrigerator. On the day of testing, the required volume of blood is removed and the cells are washed three times in normal saline. A suspension of 2.5% is made in buffered saline (pH 7.2).

Buffered saline. Buffered saline is prepared by adding 1 volume of saline to 1 volume of the buffer solution of 0.15 M  $\text{Na}_2\text{HPO}_4$  and 0.15 M  $\text{KH}_2\text{PO}_4$  mixed in the proper proportions to give the desired pH (pH 7.2 and pH 6.4).

Tannic acid. Fresh solutions of tannic acid in saline 1:20,000 are made on the day of performing the tests.

Tuberculin P.P.D. (Weybridge). This tuberculin is supplied in buffered saline (pH 7.0) with 0.5% phenol and 10% glycerol, containing 3.0 mg. of P.P.D. per millilitre. In the routine procedure 0.1 volume of this P.P.D. preparation is used in 4 volumes of buffered saline (pH 6.4) per 1 volume of 2.5% erythrocyte suspension.

Treatment of sera before test. All sera are inactivated at 56°C. for 30 minutes. One volume of serum is absorbed with 1 volume of washed packed sheep cells for 10 minutes at room temperature. The mixtures are then centrifuged for 10 minutes at 2000 r.p.m.

Test tubes. The haemagglutination tests are carried out in small culture tubes (10 x 75 mm.).

# THE HISTORY OF THE ROYAL SOCIETY OF LONDON

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Preparation of erythrocytes with tannic acid. One volume of 2.5% sheep erythrocytes in buffered saline (pH 7.2) is added to 1 volume of 1:20,000 tannic acid, the mixture is incubated for 10 minutes in a 37°C. water bath.

The tanned cells are washed once in buffered saline (pH 7.2) and centrifuged for 3 minutes at 1,500 r.p.m., and resuspended in 1 volume of saline.

Adsorption of antigens by erythrocytes. One volume of tannic acid-treated erythrocyte suspension is added to 4 volumes of buffered saline (pH 6.4) containing 0.1 volume of P.P.D. (Weybridge) (i.e. 0.15 mg. P.P.D. per ml. of 2.5% erythrocyte suspension). The mixture is incubated for 15 minutes at room temperature and then centrifuged at 1000 r.p.m. for 3 minutes. The cells are washed once in 1:250 N.R.S. and finally resuspended in 1:250 N.R.S. to the same volume. At this point gentle handling of the cells is necessary during the washing and resuspending operations.

Exposure of the cells to the test serum. The desired dilutions of the test sera are made in 1:100 N.R.S. in 0.5 ml. volumes. To each tube of the dilutions 0.05 ml. of the 2.5% suspension of tannic acid-tuberculin-treated cells is added. All tubes are shaken immediately. Satisfactory readings of the results can be made after 2 hours at room temperature, but more clear-cut results are obtained after overnight incubation in the refrigerator (2-4°C.).

The following controls are required:

- (1) 1:100 N.R.S., 0.5 ml. + 0.05 ml. sensitized cells.
- (2) Serum 1:5 dilution, 0.5 ml. + 0.05 ml. untreated cells.
- (3) Serum 1:5 dilution, 0.5 ml. + 0.05 ml. tannic acid-treated cells.













**B29769**